UiT The Arctic University of Norway

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Determinants of Staphylococcus aureus colonization and infection

Characterization of interaction between serine-aspartate containing protein D and human Siglec11 and Siglec16

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1 Contents

2 List of Tables

3 List of Figures

4 Abbreviations

1. C terminus – carboxyl terminus
2. Clf A – Clumping factor A
3. Clf B – Clumping factor B
4. CWA – Cell Wall Anchored proteins
5. DAP – DNAX activating protein
6. ddNTPs – Dideoxynucleotide Triphosphate
7. DMEM – Dulbecco's Modified Eagle Medium
8. dNTPs – Deoxynucleotide Triphosphate
9. Dsg1 – Desmoglein 1
10. FBS – Fetal Bovine Serum
11. Fnb A – Fibronectin binding protein A
12. Fnb B – Fibronectin binding protein B
13. FWD – Forward
14. GOI – Gene of Interest
15. HAIs – Healthcare Associated Infections
16. HRP – Horseradish Peroxidase
17. ICUs – Intense Care Units
18. IPTG – Isopropyl β-D-1-Thiogalactopyranoside
19. ITAM – Immunoreceptor Tyrosine-based Activation Motif
20. ITIM – Immunoreceptor Tyrosine-based Inhibitory Motif
21. KO- Knock-Out or Isogenic Mutant NCTC8325-4ΔsdrD strain of *S. aureus*
22. LA – Luria Agar
23. LB – Luria Broth
24. MSCRAMMs – Microbial Surface Components Recognizing Adhesive Matrix Molecules proteins
25. N terminus – amino terminus
26. ON – Overnight
27. PBS – Phosphate Buffered Saline
28. PCR – Polymerase Chain Reaction
29. PLL – Poly-L-Lysine
30. PMA – Phorbol 12-Myristate-13-Acetate
31. PMNs – Polymorphonuclear leukocytes

32. RCF (x g) – Relative Centrifugal Force
33. RPM – Revolutions per minute
34. RPMI – Roswell Park Memorial Institute Medium
35. RT – Room Temperature
36. RWD – Reverse
37. *S. aureus – Staphylococcus aureus*
38. Sdr – Serine aspartate repeat
39. SdrD – Serine aspartate repeat containing protein D
40. Siglec – Sialic acid binding immunoglobulin lectin
41. SOC medium – Super Optimal Broth
42. TAE – Tris acetate EDTA buffer
43. TSB – Tryptic Soy Broth
44. WT – Wild-Type or NCTC8325-4 strain of *S. aureus*

5 Summary

Staphylococcus aureus is a Gram-positive opportunistic pathogen responsible for a range of infections that can lead to fatal invasive diseases such as pneumonia or osteomyelitis. Approximately 30% of the healthy adult population are persistently colonized by *S. aureus* strains in their anterior nares. The molecular mechanism underlying *S. aureus* colonization and infection during its interaction with the host is not fully understood.

S. aureus can express several virulence determinants during its interaction with the host and/or host components. One of these virulence determinants is the serine-aspartate containing protein D (SdrD). It can increase *S. aureus* ability to survive in the blood and during systemic infections. SdrD is important for *S. aureus* colonization, survival, and infection of the host.

Unpublished results have demonstrated a possible interaction between SdrD and various proteins in human blood plasma. One of these proteins was identified as the human protein Siglec16. The biological functions of Siglec16 are mostly unknown however, studies show high identity between extracellular regions of Siglec11 and 16. Therefore, this study also includes the Siglec11 as a possible binding partner of the SdrD in the plasma.

The aim of this thesis was to develop the biological tools which will facilitate the characterization of the biological implications of the possible interaction between SdrD and Siglec16 and/or Siglec11.

The overall results show a slight progress in obtaining the tools to help in further research in characterizing the biological functions of the proteins Siglec16 and Siglec11. However, some results remain unclear and inconclusive in demonstrating a possible or significant interaction between SdrD and the Siglec proteins 11 and 16.

6 Introduction

6.1 Staphylococcus aureus

Staphylococcus aureus is a Gram-positive spherical bacterium with a diameter of approximately 1 µm (1). Its cells form grape-like clusters which appear as golden colonies on a rich medium. The golden pigmentation is a result of the presence of carotenoids. These have been described as a virulence factor that protects the bacteria against oxidants produced by the host's immune system. *S. aureus* is known as facultative anaerobe. It can produce coagulase which causes plasma to coagulate by converting fibrinogen into fibrin (2).

The genome of *S. aureus* is a circular chromosome. It has a total size of around 2.8-2.9 mega base-pairs with a (G-C) content of around 33%. The chromosome encodes approximately 2700 protein coding sequences including also structural and regulatory RNAs. It contains the core genes and other components known as 'accessory' such as foreign genes. The core genes are responsible for the housekeeping functions related to cell survival such as the synthesis of metabolic molecules, nucleic acids and replication (3). Therefore, their organization is highly conserved with an identity of 98-100% between *S. aureus* isolates. 25% of the circular chromosome is composed of the 'accessory' components which consist of mobile genetic elements. These elements are defined as DNA fragments able to encode one or more virulence determinants. They are necessary for adaptation of the isolates to environmental conditions. Some of them are pathogenicity islands (genes coding for superantigen toxins), genomic islands (genes coding for toxins and virulence factors) and phages which have the ability to horizontally transfer genes between strains (2).

S. aureus is a frequent cause of infections ranging from minor skin infections to fatal invasive diseases such as necrotizing pneumonia, osteomyelitis, and sepsis (4). *S. aureus* infections are majorly due to breaches in the host's innate immunity such as damage to the epithelial or mucosal membranes. Therefore, *S. aureus* species are referred as opportunistic pathogens (5).

6.2 *S. aureus* colonization

Primary niche of *S. aureus* in humans is the anterior nares of the nasal. Studies have shown that 20-30% of the healthy adult population are persistently colonized by *S. aureus* strains in their anterior nares. Other carriage patterns are classified as intermittent and non-carriage (6). As intermittent carriers and non-carriers have been observed to share similar ways of eliminating the pathogen, the categorization has been updated to persistent and non-persistent

carriers. Persistent carriers are usually colonized by a single strain while the non-persistent carriers tend to be colonized by different strains in specific time-periods (7).

The nasal carriage can influence the colonization of *S. aureus* in other areas of the human body such as the gastrointestinal tract (8). It is also considered a risk factor for patients that are admitted in intense-care units (ICUs) because it can cause healthcare-associated infections (HAIs) (9). Some of these infections mostly observed in hospitalized patients can be severe such as pneumonia (10).

6.3 Virulence factors of *S. aureus*

The expression of various virulence determinants is what makes *S. aureus* an efficient human opportunistic pathogen. These determinants enable the effective colonization of its ecological niches within the host, also evading the latter's innate and adaptive immune responses (4).

The virulence determinants are also known as virulence factors and are expressed on the surface or secreted into the bacterial extracellular matrix (11). Their contribution to the overall infection pathogenesis of *S. aureus* is highly significant. For example, the secretion of the membrane damaging toxins causes tissue damage and septic shock in patients (2).

Other surface factors have shown to promote adherence to cells, tissues and interact with blood components in order to evade the host's immune responses and ensure the survival of the bacteria (11).

6.3.1 Surface proteins

The surface proteins play an important role in bacterial colonization by ensuring its binding to host's cells surface proteins as well as the extracellular surface matrix. *S. aureus* contains up to 24 cell wall-anchored (CWA) proteins on its surface (Figure 6-1). Their expression depends on the strain type and growth conditions. CWA proteins have in common a signal sequence (S), a wall-spanning region (W) and a sorting signal (SS). The sorting signal present in the carboxyl terminus can be covalently anchored to the peptidoglycan of the host's cell wall with the help of sortase enzymes (12). The CWA proteins are involved in the adhesion, invasion, and evasion of the host's immune responses. Moreover, these proteins can participate in the formation of biofilms (13).

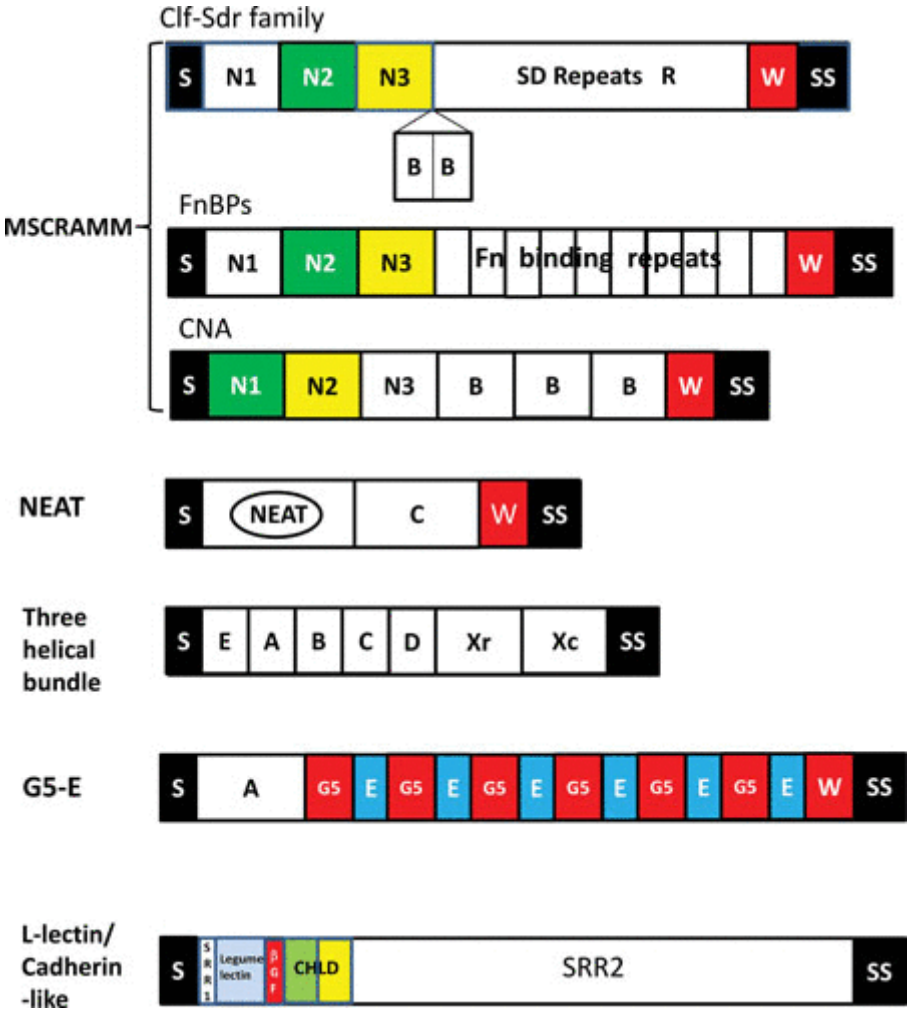


Figure 6-1. Classification of the CWA proteins based on their structural motifs. All translation products of these proteins have a signal sequence (S) at their amino terminus (N). Also, a wall-spanning region (W) and a sorting signal (SS) at their carboxyl terminus (C). Source: (12).

6.3.1.1 Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs)

(MSCRAMMs) are a subgroup of CWAs (14) as seen above in Figure 6-1. Various factors are part of MSCRAMMs such as the Sdr family members and the clumping factors A (ClfA) and B (ClfB) (15).

These proteins have been shown to initiate endovascular, bone and joint infections (8). For example, ClfA is seen responsible for the clumping of *S. aureus* in blood plasma and a virulent for causing septic arthritis and infective endocarditis in mice and rabbit models (16).

Their structure can bind to host molecules like collagen, fibronectin (via FnbA and FnbB) and fibrinogen (via ClfA and ClfB) (17). This ability, to bind to several host molecules enables them to evade the host's immune system and develop infections (18).

6.3.1.2 Serine-Aspartate Repeat proteins

Sdr proteins have a characteristic feature called the R region which contains several serine-aspartate repeats (19). The *sdr* locus encodes three proteins: SdrC, SdrD and SdrE. The *sdrC* gene is always present in the locus however, all three genes are not always present in all *S. aureus* strains (20).

A study made on SdrC shows that this protein can bind to the neuronal protein β-neurexin 1 (21). SdrE has been implied to capture a complement H factor on the surface of *S. aureus* as a mechanism to evade the host's immune system (22). While SdrD is shown to be crucial for abscess formation (23).

6.3.2 Serine-Aspartate Repeat containing protein D (SdrD)

SdrD belongs to the *sdr* genes family. They have been observed in many *S. aureus* isolates (24). A study aiming to evaluate the allelic variation of the *sdrD* gene among *S. aureus* from healthy carriers has provided evidence of sequence variations across different domains of *sdrD* gene. The N2, N3 and B1 subdomains in the gene are diverse however all the other regions appear to be conserved in *S. aureus* isolates (25).

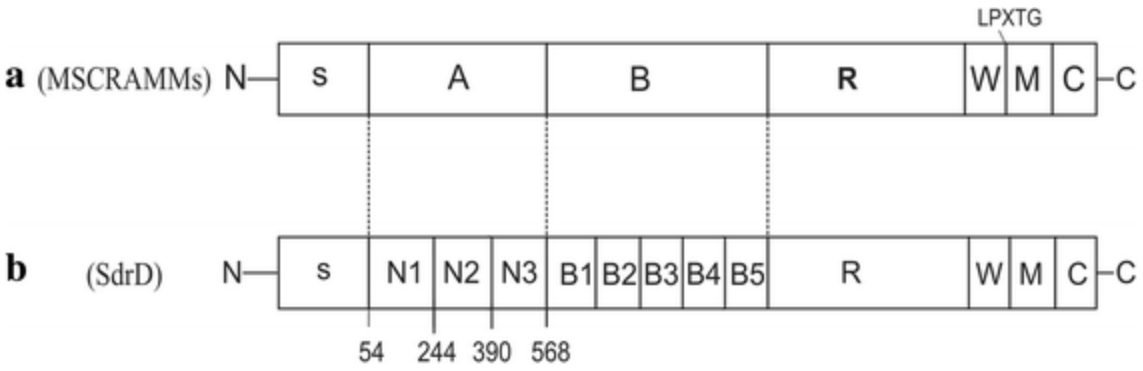


Figure 6-2. A schematic representation of the structures of (a) MSCRAMMs and (b) sdrD gene from S. aureus NCTC8325. (S) is the signal sequence region; (A) is the region responsible for ligand binding which in scheme (b) is divided into three subregions N1-N3; (B) is the region which contains the EF-motifs and is divided in 5 subregions as shown in scheme (b); (R) is a serine-aspartic acid repeat region; in the C-terminus there are three regions marked as (W) the wall-spanning region, (M) the transmembrane region and (C) the cytoplasmic region; the LPXTG motif is present between (W) and (M) regions. Source: (25).

The different regions in the *sdrD* structure contribute to its function (Figure 6-2). The signal sequence (S) can direct the protein to different locations within the peptidoglycan of the bacterial cell wall. *S. aureus* can distinguish between the presence or absence of a specific motif in the signal peptide and decide the specific location of the protein (26). The A region is the

ligand binding region. It is divided into three subdomains N1, N2 and N3. A is known to be a fibrinogen interactive domain (27). The B repeat is made up of five subdomains B1-B5 where each of them contains one conserved EF-hand motif. These EF-motifs can interact with calcium ions (28).

SdrD is recognized as an important virulence factor of *S. aureus* as it can increase its ability to survive in the blood and during systemic infections (29). *S. aureus* strains that are reported to cause bone infections have a higher prevalence of the expression of the *sdrD* gene (30).

SdrD expression has shown to promote bacterial adhesion to desquamated nasal epithelial cells (7) and contribute to abscess formation (23). In addition, desmoglein1 (Dsg1) has been identified as a host ligand for SdrD protein and their interaction has also proven to promote bacterial adhesion to host cells (31).

Therefore, according to these observations, the overall contribution of SdrD protein is shown to be significant to *S. aureus* colonization, survival, and infection of its host.

6.4 *S. aureus* against the immune cells: Macrophages

Macrophages are professional phagocytes and an important component of the innate immune response equipped with antimicrobial effectors. *S. aureus* can successfully establish and maintain infection by evading the macrophage dependent killing (32). Macrophages can present antigens from the fluid phase uptake or from phagocytosed microbes. They have the capacity to control microbial infections however, they fail to eradicate *S. aureus*. When macrophages fail to eliminate *S. aureus*, the bacteria can coordinate the expression of virulence determinant genes. These proteins can then affect the functions of other immune cells and stop opsonization and phagocytosis (4).

6.5 *S. aureus* against the immune cells: Neutrophils

Polymorphonuclear leukocytes (PMNs or Neutrophils) are a critical component of innate immunity and essential in controlling bacterial infections. Their presence in a specific area is known as a hallmark for *S. aureus* infection (33).

They are the first line of defense against bacterial pathogens that have breached epithelial barriers. Neutrophil response includes the migration following the gradient of chemokines and cytokines guiding them to the site of infection where they ingest microbes (34). They can expose microbes to antimicrobial proteins, peptides, and reactive oxygen species to kill them.

They can also secrete cytokines/chemokines to continue the recruitment of other neutrophilic cells and subsequently, enhance several host responses against the bacterial infection. Their death is essential for eradicating the infection and should be regulated to minimize tissue damage (35).

S. aureus during infection can survive intraphagosomally without multiplying however, they produce factors that lyse neutrophils, resulting in pathogen survival and promotion of pathogenesis and disease (36). After *S. aureus*-neutrophil interaction, the bacteria can also produce proteins that are involved in virulence, metabolism, and capsule synthesis of *S. aureus* (37).

6.6 Sialic acid-binding immunoglobulin-type lectins (SIGLECs)

Siglecs are a family of 15 human and 9 murine cell surface receptors that are expressed in white blood cells of the immune system (38). They can recognize ligands known as determinants of 'self' and bind to glycans of glycoproteins and glycolipids of cell surfaces present in all mammalian cells that contain sialic acid (39).

All members of the Siglec family (Figure 6-3) have a common N-terminal known as the 'V-set' immunoglobulin domain that binds ligands that contain sialic-acid. The immunoglobulin domains known as the 'C2-set' vary among members of the family. This set can extend the ligand-binding site away from the membrane surfaces (40).

Tyrosine motifs are commonly present in many of the Siglecs. Other motifs found in them are the immunoreceptor tyrosine-based inhibitory motifs (ITIM) and ITIM-like motifs. Co-receptors involved in regulation of cell-signaling usually contain these type of motifs (41).

Some Siglecs such as Siglec16 are characterized by the absence of the tyrosine motifs, however they seem to contain a transmembrane spanning positively charged region that can associate the Siglecs with adaptor proteins such as DAP-12 (the DNAX-activating protein) which is known to give positive and negative signals (42).

Sialylated ligands can interact with Siglecs resulting in modulating activities in cell signaling and cell-cell interactions. Some members of this family have demonstrated to bind pathogen that carry sialylated glycans mimicking 'self' thus, downregulating the initiated host's immune responses against them (39). The excessive expression of sialic acid in tumor microenvironments has been connected with immune suppression which suggest that using

Siglecs as a target can result in beneficial effects in cancer therapy, autoimmune diseases, and allergies (43).

As mentioned, the human genome contains 15 different Siglecs. These are divided into two groups according to their genetic homology among mammalian species. The 1st group seems to be present in all mammals and involves the following members: Siglec-1, -2, -4 and -15 whereas the 2nd group involves the Siglecs related to CD33 which are Siglec-3, -5, -6, -7, -8, -9, -10, -11, -14 and -16. The latter group members have been rapidly evolved and their presence between species differs (43).

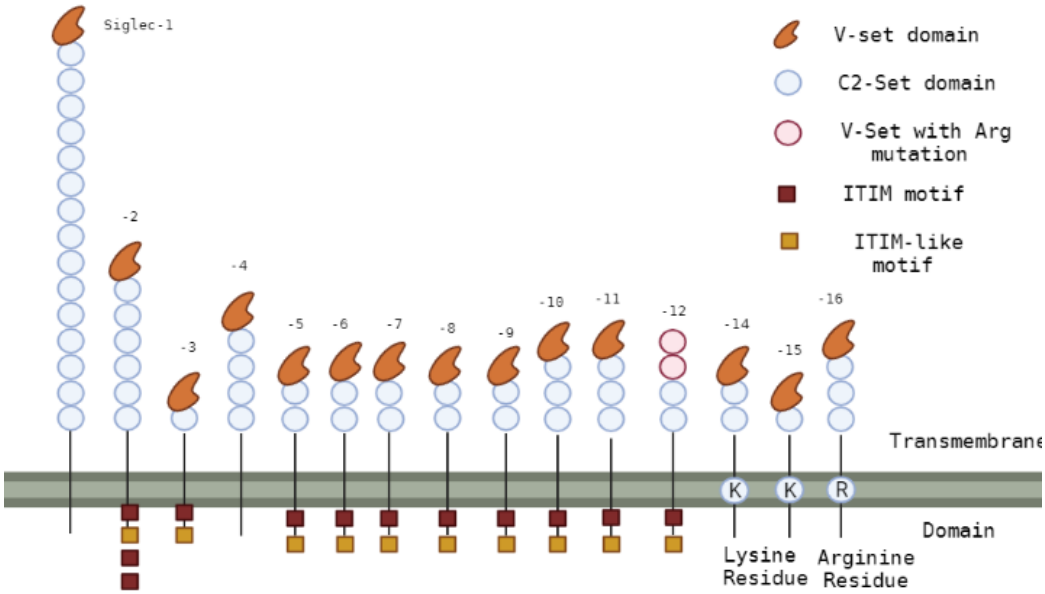


Figure 6-3. Illustration of the human Siglec family receptors. They carry an extracellular constant V-set domain that binds with sialic acid ligands and various numbers of C2-set domains. Siglec12 presents an exception as it contains two constant domains with the absence of arginine therefore, it cannot bind with sialic acid ligands. Most have ITIM and ITIM-like motif domains that facilitate inhibitory signals to the cells. Siglec-14, -15 and -16 have positively charged residue in the transmembrane domain (lysine and arginine) that recruit DAP-12 and facilitate activating signals to the cells. Adapted by: (44).

6.6.1 Siglecs: Biological functions

Biological functions of Siglecs have been analyzed in genetically modified mice models. Deficiency of Siglec4 lead to non-stable myelin sheaths (40); while the genetic deletion of Siglec2 resulted in extreme calcium response by B cell cross-linking receptors. It also affected the biology of these cells resulting in various changes. Phenotypes of different models for the Siglec2 have appeared inconclusive depending on the experimental conditions and the genetic

background of the animal models. Other Siglecs behaviors such as -1 and -3 have also been studied however the results remained inconclusive (40).

The second group of Siglecs, the ones related to the CD33, have been challenging to study in vivo due to their difference between humans and mice models. Therefore, functional studies of human Siglecs require the use of cell lines, primary cell cultures or even freshly isolated cells from patients and healthy donors (45).

6.6.2 Siglecs: Therapeutic targets

Immune therapeutic studies have targeted Siglecs, as a treatment therapy due to their highly restricted expression in the immune system. Moreover, they seem to have differential expression by various immune cells such as B cells or NK (natural killing) cells. Siglecs are recognized as endocytic receptors. This makes them good candidates for targeted therapeutics to deliver cytotoxic and immune modulators into cells (46).

6.6.3 Siglec11 and Siglec16: Evolution

Siglec-11 and *-16* genes are located in a head-to-head orientation around 1 Mb away from the *CD33-related Siglec* gene cluster in humans. Human *Siglec16* lies around 9 kb 5' of *Siglec11* on chromosome 19q13.3 (47). Studies have shown the loss of this gene in some species and its duplication in other ones consequently, creating 2 head-to-head ITIM containing genes. It is suggested that *Siglec11* underwent conversion in the 3' portion losing its ITIM domain and gaining a sequence that is capable of recruiting an immunoreceptor tyrosine-based activation (ITAM) domain. This newly formed gene then underwent pseudogenization in the human lineage and is nowadays known as *SiglecP16* (48). *SiglecP16* is recognized as a null allele of a functional *Siglec16* gene that is seen in some humans showing a low allele frequency of about 0.1 to 0.3 in a worldwide population study (47).

The human *Siglec11* gene has shown reduction in sialic acid binding abilities in comparison with its ancestral form. Moreover, it seems to recognize oligo-sialic acids which are enriched in the brain. It also seems to be expressed in extra-neural tissue macrophages in humans (49). The antibody for Siglec11 can recognize the expression of Siglec16 in the brain. The latter shows an absence of the cytosolic signaling motif but instead it can interact with the transmembrane adaptor molecule DAP-12 with a cytosolic activating ITAM motif (50).

Siglec-11 and -16 have identical ligand binding domains and are expected to bind to the same set of ligands in the brain, but it has been noticed that once they activate, they show opposite

inflammatory responses. Siglec11 can activate phosphatases such as SHP-1 and SHP-2 via ITIM whereas Siglec16 can recruit activating kinases such as Syk and ZAP-7 via ITAM which is absent in ~60% of the homozygous humans for *Siglec16*. Due to their highly similar extracellular domains, there is no present reliable way that can distinguish between human Siglec-11 and -16 using immune-histochemical methods (47).

6.6.4 Siglec-11*:* Characteristics and function

Siglec11 belongs to the CD33-related Siglecs group. It contains a cytosolic domain phosphorylated at tyrosine residues which can bind to protein-tyrosine phosphatases (SHP-1 and SHP-2) and more specifically to α2-8-linked sialic acids. It is absent on peripheral blood leukocytes however its expression is observed on macrophages in various tissues such as Kupffer cells and brain microglia (38).

An extreme high nucleotide sequence identity has been observed between *Siglec11* and *SiglecP16* thus, it is thought that the latter is very likely to be an ancestor of *Siglec11*. It is suggested that the extracellular domain was duplicated from the functional ancestor of *SiglecP16* as it is noticed an identity of about 97% of the *SiglecP16* pseudogene and *Siglec11* in this particular region (51).

The expression of Siglec11 has been observed in human brain tissue which can limit damage by innate immune cells during homeostasis or brain inflammation. It has been shown to mediate anti-inflammatory signaling, inhibit phagocytosis and lessen neurotoxicity of microglia by interacting with poly-sialic acid (PSA) on neurons (52). The current knowledges can indicate new possible therapeutics interventions of microglial Siglec11 in neurodegenerative diseases (53).

6.6.5 Siglec16: Characteristics and functions

Siglec16 was originally reported to be non-functional based on the 4bp deletion in the second exon that encodes for the first N-terminal immunoglobulin-like domain. A genetic analysis made on UK Caucasians conclude that this protein is not a pseudogene and that it can encode a full ORF. Moreover, the analysis made on polymorphism revealed a 50-50% split in the UK population between the wild-type allele and the 4bp deletion allele (52).

Its highly compact in the human genome, devoid of repetitive elements in the coding region. This can suggest the limitation of DNA recombination between coding regions and the extracellular domain as they are highly conserved among Siglecs (more specifically in Siglec-

10, -11 and -P16) (38). According to some studies, *Siglec16* is described as being evolved from *Siglec11* as it is also associated with DAP-12 which is expressed in macrophages. The Siglec16 transmembrane domain contains a charged lysine residue, and it has a short cytoplasmic tail without ITIM motif. Siglec16 protein appears with a molecular wight of around 58 kDa (47).

Siglec16 is paired with Siglec11, sharing a 99% homology in their first three extracellular immunoglobulin superfamily domains (52). Engagement of Siglec16 in the brain with the same ligand as Siglec11 can however trigger inappropriate immune and inflammatory responses.

Overexpression of Siglec16 in human THP1 macrophage-like cell lines has been shown to suppress the production of pro-inflammatory cytokines, tumor necrosis factor alpha and interleukin-6 while increasing interleukin-10 production which is an anti-inflammatory cytokine (52).

6.7 Combating *S. aureus*

The development of antibiotic-resistant *S. aureus* has increased the need to control its infection (11). The understanding of its defense mechanism to evade host defenses are increased, however, no successful vaccine has been obtained so far. A therapeutic maneuver is thought to be enhancing the killing capacity of neutrophils after pathogen uptake. Novel approaches are targeting virulence factors as it is believed that a combination of therapeutic approaches that include targeting the most important virulence determinants is required for combating *S. aureus* (36).

Thus, a vaccine that simultaneously targets multiple *S. aureus* virulence factors is needed and encouragingly some are in the pipeline. Moreover, targeting sensory/regulatory systems has great potential for therapeutic and vaccine development. Theoretically, chemical inhibitors and neutralizing antibodies of sensory/regulatory systems could inhibit *S. aureus* from sensing its environment and producing virulence factors that disrupt host antimicrobial functions (34).

The complex adaptation of *S. aureus* to many different host niches is also considered when designing vaccines against this pathogen (54). It is suggested that a rational and logical approach to vaccine development must simultaneously address multiple virulence and bacterial defense mechanisms. However, how many, and which antigens should be targeted by a vaccine is a matter of some debate (55).

7 Aims

Previous studies have shown that the expression of SdrD is upregulated and increases bacterial survival within human blood (19) (29). In order to go further into the mechanism behind this, recombinant SdrD was used as bait to pull down interaction partners in human blood. Among the putative targets were the Siglecs {Ajayi et al, unpublished result}. The aim of this thesis was to create tools to verify/dispose the interaction between SdrD and the Siglec proteins and to evaluate the purpose of the interaction. In order to achieve this, the specific aims were as follows:

1. To clone full length human Siglec16 (hSiglec16 full) and extracellular region (hSiglec16 extra) in prokaryotic expression vector of pT7-Flag-2 and in the eukaryotic expression vector pDEST YFP
2. To express hSiglec16 full and hSiglec16 extra in *E. coli* Lemo21 (DE3) for purification of the recombinant proteins
3. To transfect HEK293-TLR2 with pDEST YFP:hSiglec11 full or pDEST YFP:hSiglec16 full to evaluate effect of SdrD and Siglecs (-11 and -16) interaction on adhesion, internalization and survival assays as well as on cytokine response

8 Materials

Detailed information and the complete list of the materials needed/used in this study is provided in the tables below.

Table 8-1. The list of bacterial and mammalian cells

Identity	Specificity	Provided by/Originated from
***E. coli* DH5α**	Competent bacterial cells	New England Biolab inc/
***E. coli* Lemo21 (DE3)**	Competent bacterial cells engineered for transmembrane protein expression	New England Biolab inc.
***S. aureus* NCTC8325-4**	*S. aureus* strain	*S. aureus* NCTC
***S. aureus* NCTC8325-4 ΔsdrD**	*S. aureus* strain where SdrD gene was knocked-out	(31)
HEK293-TLR2	Human Embryonic Kidney cells	Invivogen
THP1	Immortal Monocyte-like cell line	Sigma-Aldrich

Table 8-2. Expression vectors used to build the desired constructs

Expression Vectors	Type	Selective Antibiotic (concentration)	Procedure	Provided by/Purchased from
pT7-Flag-2	Prokaryotic	Ampicillin (100 µg/ml)	Cloning of hSiglec16full and hSiglec16extra	Sigma-Aldrich
pDONR221	'donor vector'	Kanamycin (50 µg/ml)	BP reaction of Gateway cloning of hSiglec11 and hSiglec16	Kind gift from Dr. Yakubu, IMB, UiT
pDEST-YFP	Mammalian 'destination' vector	Gentamycin (20 µg/ml)	LR reaction of Gateway cloning of hSiglec11 and hSiglec16	Kind gift from Dr. Yakubu, IMB, UiT

Table 8-3. The list of primers and the corresponding procedures

Primers	Type	Sequence (5'-3')	Procedure	Purchased from
hSiglec16 full	Forward (fwd)	TAAACAAAGCTTATGCTGCTGCTGCCCCTGCTGCT	Cloning: PCR	Sigma
hSiglec16 full	Reverse (rwd)	TGCTTAGAATTCTTAGTCCGTGACAGCGTCTGC	Cloning: PCR	Sigma
hSiglec16 extra	Forward (fwd)	TAAACAAAGCTTAACAAGGATCCCAGTTACAGT	Cloning: PCR	Sigma
hSiglec16extra	Reverse (rwd)	TGCTTAGAATTCTCCCAGCGGGTGCCGA	Cloning: PCR	Sigma
T7 promoter	Forward (fwd)	CTATCATGCCATACCGCGAAAGG	Sequencing PCR	Sigma
hSiglec11 attB1	Forward (fwd)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGGCCGCCACCATGGTCCC	Gateway Cloning: PCR	Sigma
hSiglec11 attB2	Reverse (rwd)	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTGGAACCATCCCTGACATCTCCCT	Gateway Cloning: PCR	Sigma
hSiglec16 attB1	Forward (fwd)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGGCCGCCACCATGCTGCT	Gateway Cloning: PCR	Sigma
hSiglec16 attB2	Reverse (rwd)	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCCGTGACAGCGTCTGCAGC	Gateway Cloning: PCR	Sigma
CMV promoter	Forward (fwd)	CGCAAATGGGCGGTAGGCGTG	Sequencing PCR	Sigma

Table 8-4. Reagents and kits

Product	Procedure	Provided by/Purchased from
NucleoBond Xtra Midi	Plasmid Isolation	Takara Bio Company
High Fidelity Phusion	Polymerase Chain Reaction (PCR)	Thermo Fisher
Agarose Powder	Gel Electrophoresis	SeaKem® LE
TAE Buffer	Gel Electrophoresis	Invitrogen
GelRed	Gel Electrophoresis	Biotium
6X Loading Buffer	Gel Electrophoresis	Thermo Fisher
1kb+ molecular ladder	Gel Electrophoresis	Invitrogen
Monarch PCR/DNA clean up	Purification of PCR products	NED. Inc
Monarch DNA gel extraction	Extraction of products from agarose gel	NED. Inc
DreamTaq	Colony PCR	Thermo Fisher
BigDye 3.1	Sanger Sequencing	Thermo Fisher
B-PER reagent	Protein extraction from bacterial cells	Thermo Fisher
Coomassie Plus (Bradford)	Protein Concentrations	Thermo Fisher
Bench Marker	SDS-Page	Thermo Fisher
Magic Marker	Western Blot	Thermo Fisher
SeeBlue	SDS-Page/Western Blot	Invitrogen
Nitrocellulose membrane	Western Blot	Sigma Aldrich
BP clonase II Mix	Gateway cloning	Thermo Fisher
LR clonase II Mix	Gateway cloning	Thermo Fisher
Metafectene Pro	Transfection of eukaryotic cells	Biontex
CytoTox 96® Non-Radioactive	Cytotoxicity assay	Promega
Human IL-6 ELISA	IL-6 concentration	Thermo Fisher

9 Methods

9.1 Cloning of Siglec16 full length and Siglec16 extracellular domain into pT7-Flag-2 prokaryotic vector

Cloning is considered a set of experimental techniques that generate a population of organisms carrying the same molecule of recombinant DNA. The DNA is firstly assembled in vitro and then transferred into a host organism (usually the easy-to-grow, nonpathogenic laboratory bacterial strain of *Escherichia coli*) that directs its replication in coordination with its growth. (56)

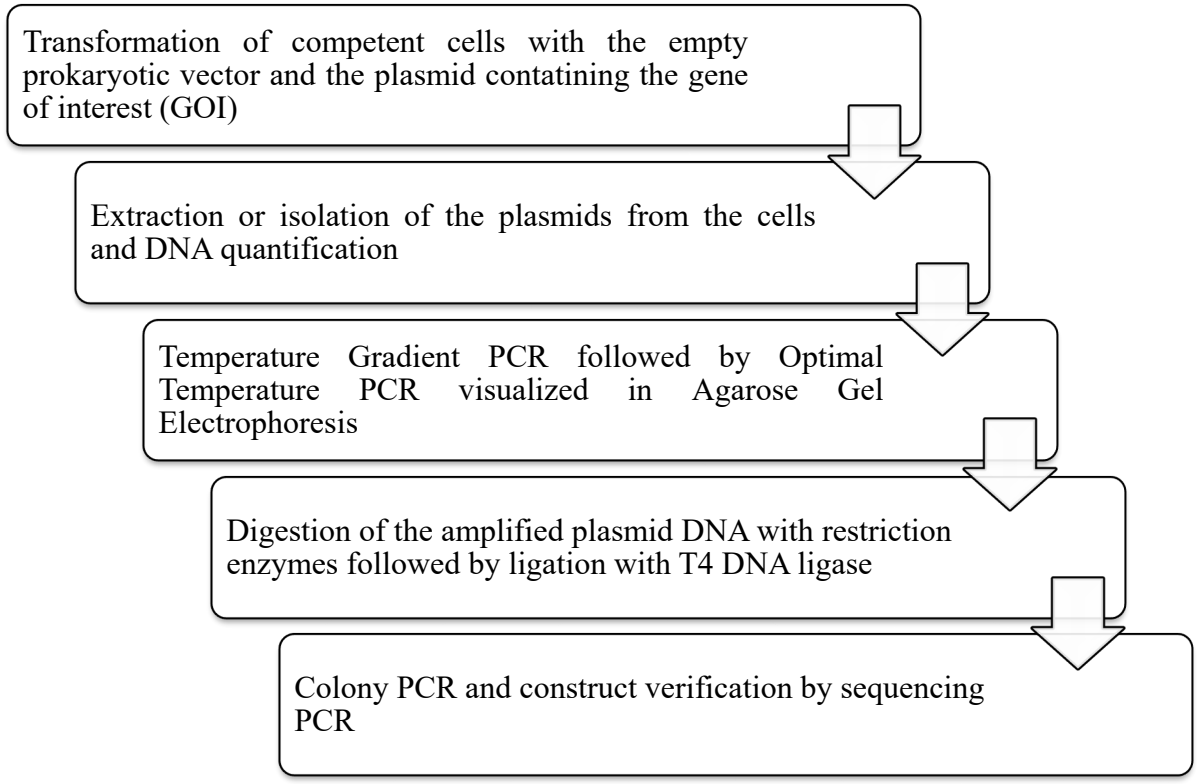


Figure 9-1. Schematic representation of the set of experimental techniques of traditional cloning. Each individual process and corresponding protocol are explained in detail further on.

9.1.1 Transformation of competent cells

Competence for genetic transformation is a physiological state that enables the uptake of exogenous DNA (57). The ability of the cells to uptake foreign DNA from their environment makes them competent while the actual process is known as transformation.

Transformation is otherwise described as the ability of bacteria to introduce individual molecules of plasmid DNA into their cells (58). Transformation can naturally occur in bacteria however artificial methods have been optimized to increase the uptake and expression of target DNA through heat-shock, chemical, electro etc. Efficacious competent cells commonly used in cloning applications are the *E. coli* DH5α. The transformants carrying the plasmid that contains the gene of interest (GOI), are grown in media with antibiotic. The plasmids containing the GOI have a resistance gene against a certain antibiotic which helps them survive in a growth media with that antibiotic.

9.1.2 Transformation of DH5α competent cells

Tubes containing the competent DH5α cells, the empty pT7-Flag-2 prokaryotic vector and the plasmid containing the DNA of *Siglec16* were kept on ice during the process. In four new Eppendorf tubes marked pT7-Flag-2, pSiglec16 full, pSiglec16 extra and negative control, 50 µl of competent cells were added. 1 µl of corresponding plasmid DNA was pipetted into the tubes. The mixes of competent cells and plasmid DNA were incubated on ice for 30 minutes. After incubation time, the cells were heat shocked at 42ºC for 90 seconds to facilitate the uptake of the plasmid DNA. The cells were put on ice for 90 seconds after the heat shock. 200 µl of SOC recovery broth was added to the cells which were incubated at 37ºC with shaking 225 rpm for 45 minutes. Three different volumes were plated out on Luria Agar (LA) plates containing 100 µg/ml ampicillin from each of the tubes: 25, 100 and 125 µl. The plates were incubated at 37ºC overnight.

9.2 Preparation of overnight cultures and preservation of transformed bacteria

Preparation of overnight cultures of a transformed colony of the *E. coli* aims to increase the numbers of cells that have taken up the exogenous target DNA during transformation. Target plasmid DNA needs to be isolated from the cultures for further analysis. The bacteria are grown in a culture media that contains a selective antibiotic corresponding to the resistance gene present in the exogenous plasmid DNA to ensure that only the transformed bacteria can survive. In this study the selective antibiotic for the prokaryotic vector and the target plasmid DNA was ampicillin (mentioned in the instructions that come with the purchased product).

Transformation efficiency was impossible to calculate due to the large number of colonies that grew on the plates the next day. Single colonies from the 100 µl volume plates were picked out for the making of the overnight cultures. Same colonies were plated out in a fresh plate to make freezing stock the next day to preserve bacteria containing the plasmids of interest. For the

overnight cultures, 100 ml of Luria Broth (LB) media with 100 µg/ml ampicillin was pipetted in 250 ml flasks. The single colonies were transferred using a loop into the corresponding marked flask, one for the empty prokaryotic vector, one for the pSiglec16 full and another for pSiglec16 extra.

9.3 Isolation of the plasmids from the overnight cultures

Isolation of the target plasmid DNAs is performed to amplify and purify the targets. This, to obtain a high concentration of the plasmid which is needed in further steps of the cloning procedures such as ligation. NucleoBond Xtra Midi kit from Takara Bio Company was used for the process of plasmid extraction from the overnight cultures. The manual of the kit was followed step by step for the extraction (59).

The overnight cultures were transferred in tubes and centrifuged at 6000 x g for 15 minutes at 4ºC. The supernatant was removed, and the pellet was resuspended in 8ml RES buffer + RNase A by pipetting the solution up and down combined with mixing using a vortex until no clumps were visible. 8ml of LYS buffer was added to the suspensions and mixed gently by inversion 5-6 times before incubating for 5 minutes at room temperature. EQU buffer (12 ml) was used to equilibrate the NucleoBond Xtra columns together with the inserted filter. The columns were emptied by gravity flow. 8 ml of NEU buffer was added to the suspensions and mixed gently by inversion as previously mentioned until the blue samples became completely colorless. The lysates need to be homogenous prior applying them to the column filters to avoid clogging. After lysate filtration the column filters were washed with 5 ml of EQU buffer before they were discarded. 8 ml of WASH buffer was used to wash the columns. The plasmid DNAs were eluted into clean 15- or 50-ml Falcon tubes with 5 ml ELU buffer. The ELU buffer was preheated to 50ºC prior elution to improve the yielding of the DNA. 3.5 ml of room temperature isopropanol was added to the samples to precipitate the eluted DNAs. Tubes were vortexed thoroughly before centrifugation at 15000 x g for 30 minutes at 4ºC. After discarding the supernatants, 2 ml of 70% of room temperature ethanol was added to the pellet. Samples were recentrifuged at 15000 x g for 5 minutes at room temperature. Supernatants were discarded carefully using a pipet, and the pellets were dried at room temperature. Finally, the DNAs were dissolved in 30 µl sterile water.

9.4 DNA quantification with nanodrop

DNA quantification can determine the average concentration of a sample needed in further downstream procedures. The Nanodrop 2000/2000c Spectrophotometer (ThermoFisher) was the equipment used to quantify concentration and purity of the plasmid DNAs.

Firstly, the Nanodrop 2000/2000c software was used for the measurement of nucleic acids. Before blanking the machine with the elution buffer used to elute the plasmid DNAs, the loading plaques of the nanodrop were cleaned. After blanking, 1 µl of one of the samples was loaded and measured at a wavelength of 260 nm. The ratio of nucleic acids absorbance maximum to the absorbance at 280 nm was used to measure the purity of the extractions. If the ratio 260/280 is approximately 1.8 the DNA extractions are accepted as pure. Between each measurement, the plaques of the nanodrop were cleaned before the next sample was loaded.

9.5 Polymerase Chain Reaction (PCR)

PCR is a method which uses the ability of polymerases to synthesize new complementary strands with the template strands present in the reaction. It is considered a fast and inexpensive technique used for amplification. It has become a necessary step in almost all molecular and genetic analyses. During the PCR, the samples are firstly heated to denature template strands into single strands. The polymerase synthesis new strands complementary with the template ones using the primers (sequences that recognize and bind to template strands to initiate the amplification of the target fragment) and the dNTPs (deoxynucleotide triphosphate) present in the reaction tube. The process is repeated in generally 25-30 cycles generating millions of target copies (60).

The polymerase used in the PCR reactions in this study was the High-Fidelity Phusion polymerase (ThermoFisher). According to the manufacturer, high-fidelity is the ability of the polymerase to accurately replicate a template. The Phusion is considered to achieve a high speed and accurate PCR. It offers 50x higher fidelity compared to Taq polymerase, resulting in maximal reaction success with minimal optimization.

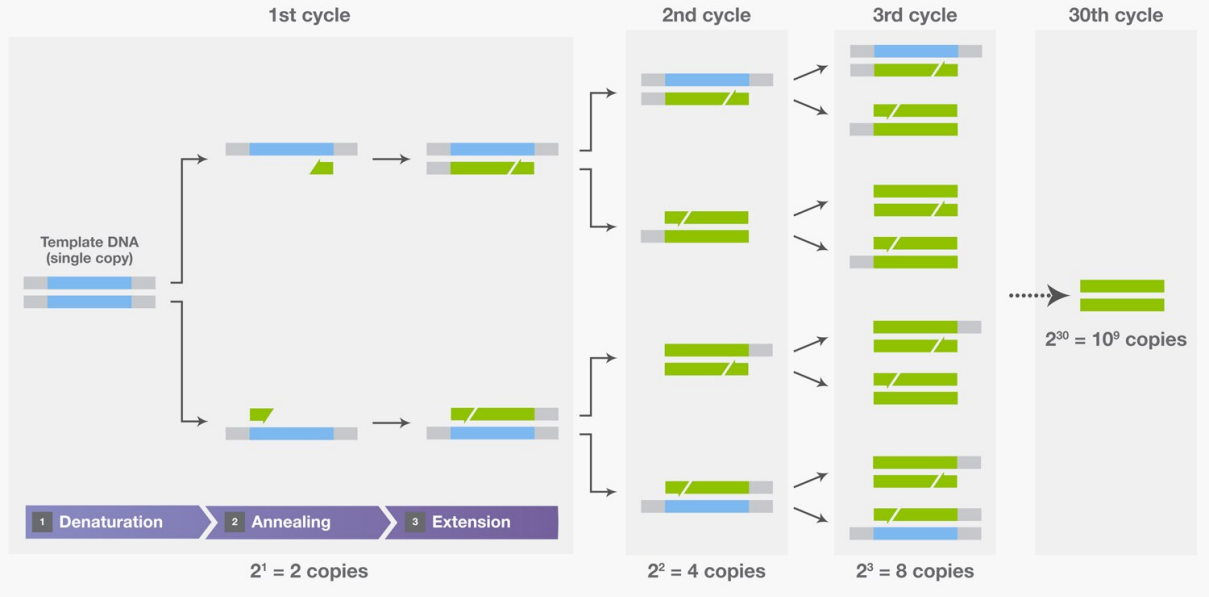


Figure 9-2. Schematic presentation of PCR cycles. 1st cycle includes the denaturation of DNA into single strands, the annealing of DNA with the specific primers added to the reaction tube and their extension using dNTPs. The complementary rule of creating a new strand from a template one results in more copies of the DNA of interest. More cycles will produce many DNA copies. Source: ThermoFisher/PCR Basics

9.5.1 Temperature Gradient PCR

The temperature gradient PCR was performed to find the optimal temperature of amplification for the DNA fragments of interest: the *Siglec16* gene and the extracellular region of the *Siglec16* gene. The PCR protocol for Phusion High-Fidelity DNA polymerase was followed for the procedure (61). The setup for the reaction is shown in Table 9-1.

Table 9-1. 20 µl PCR setup

Compounds	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**	**Control 1**	**Control 2**
5x Phusion Buffer	4 µl	4 µl	4 µl	4 µl	4 µl	4 µl	4 µl	4 µl
10 mM dNTPs	0.4 µl	0.4 µl	0.4 µl	0.4 µl	0.4 µl	0.4 µl	0.4 µl	0.4 µl
10 µM FWD	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
10 µM RWD	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
Plasmid DNA (1pg-10ng)	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	-	1 µl
Phusion	0.2 µl	0.2 µl	0.2 µl	0.2 µl	0.2 µl	0.2 µl	0.2 µl	-
Nuclease-free water	to 20µl	to 20µl	to 20µl	to 20µl	to 20µl	to 20µl	to 20 µl	to 20 µl

The samples were mixed gently, and all the liquid was collected to the bottom of the tube by a quick spin. The thermocycling conditions for the PCR were as shown in Table 9-2 below.

Table 9-2. Thermocycling conditions for the PCR

Step	Temperature (Degrees Celsius)	Time
Initial Denaturation	98	30 seconds
	98	10 seconds
30 cycles	55 – 70	30 seconds
	72	1 minute
Final Extension	72	10 minutes
Hold	4 – 10	Hold

9.5.2 Optimal Temperature PCR

Amplification of the DNA fragments of interest (*Siglec16* full and *Siglec16* extra) were performed at an optimal annealing temperature according to the results of the previous temperature gradient PCR reaction. 6 samples were prepared with a total reaction volume of 50 µl, as shown in Table 9-3. Thermocycling conditions of the reaction were as in Table 9-4.

Table 9-3. 50 µl PCR setup. All compounds were mixed and centrifuged prior to use.

Compounds	Sample 1	Sample 2	Sample 3	Sample 4	Control 1	Control 2
5x Phusion Buffer	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
10 mM dNTPs	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
10 µM FWD	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
10 µM RWD	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Plasmid DNA (1pg-10ng)	1 µl	1 µl	1 µl	1 µl	–	1 µl
Phusion Polymerase	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl	–
Nuclease-free water	To 50 µl	To 50 µl	To 50 µl	To 50 µl	To 50 µl	To 50 µl

Table 9-4. Thermocycling conditions for optimal temperature PCR. (S16) is the *Siglec16* full DNA and (S16 extra) is the *Siglec16* extra DNA.

Step	Temperature (Degrees Celsius)	Time
Initial Denaturation	98	30 seconds
	98	10 seconds
30 cycles	67 (S16) | 65.7 (S16 extra)	30 seconds
	72	1 minute
Final Extension	72	10 minutes
Hold	4 – 10	Hold

9.6 Agarose Gel Electrophoresis

Agarose Gel Electrophoresis is a separation method commonly used to analyze biomolecule fragments which are detectable by their size ranging from 100bp to 25kb. The gel consists of pores that help in organizing the molecule fragments based on charge, size, and shape. These fragments migrate to the positively charged anode due to the application of an electric current (DNA negatively charged due to the phosphate groups). Smaller fragments move more quickly than larger ones. A weight molecular ladder that contains fragments of known size is used to estimate the size of the unknown fragments. Ultraviolet light illuminates the fluorescent nucleic acid dye bound to the DNA in the unknown samples and makes the DNA fragments visible on the agarose gel (62).

Firstly, 1% agarose gel was prepared by mixing in a glass bottle 3g agarose (SeaKem® LE Agarose) with 300 ml of TAE buffer (Invitrogen). The solution was microwaved and cooled down to approximately 50ºC before use. To visualize DNA fragments under UV light, 5 µl of GelRed (Biotium) was added to 100 ml of the 1% agarose gel and poured into a 15 samples chamber under the hood. After 10 to 15 minutes the gel was polymerized and ready for use. 4 µl of 6x loading buffer (ThermoFisher) was added to all the PCR samples which were then mixed by pipetting up and down. The loading buffer was used to track the migration of DNA during electrophoresis. 12 µl of sample mix was loaded into each well. A 1kb+ weight molecular ladder (Invitrogen) (5 µl) was loaded in two different wells. The top that connects the plastic holder to the voltage machine was properly placed before grading the machine to 110 volts for 45 minutes up to 1 hour.

9.7 Purification of PCR products: Siglec16 full and Siglec16 extra

Purification of PCR products is needed to get rid of all the reagents used during the reaction which can be primers, dNTPs, buffer etc. that might interfere in downstream experiments. Purification procedure ensures a clean sample of the target DNA.

The kit Monarch PCR and DNA clean-up (NEB inc.) was used to purify the amplified DNA of pSiglec16 full and pSiglec16 extra from the PCR reaction according to the manufacturer's protocol (63). All the centrifugation steps were carried out at 16 000 x g (~ 13 000 rpm). Firstly, all the PCR samples were collected in a single tube for each of the DNAs. Binding buffer was added to dilute the collected samples and mixed well using a pipette and avoiding using a vortex. The samples were then loaded in a column and spun down for 1 minute. The flow-through after the spin was discarded. 200 µl of wash buffer was added to the column and

centrifuged for 1 minute. The flow-through was discarded after the centrifugation and this step was repeated. After washing the samples, the column was transferred into a clean 1.5 ml microfuge tube. Elution buffer (20 µl) was added to the center of the matrix and the microfuge tubes were centrifuged for 1 minute. The DNA concentration was determined using nanodrop.

9.8 Digestion of the pT7-Flag-2 empty vector and the purified PCR products using restriction enzymes

Restriction digestion using two different enzymes is a technique generally used in cloning applications that cuts the plasmid DNA in two positions in order to ligate the target gene fragment into the expression vector. To facilitate the process, it is wise to use the same restriction sites in expression vectors and the plasmids/PCR products that contain the insert DNA.

The empty prokaryotic vector pT7-Flag-2 (Figure 9-3) and the purified PCR products hSiglec16 full and hSiglec16 extra were digested using two restriction enzymes: HindIII-HF and EcoRI-HF. The reaction was set up according to the NEB-cloner protocol from BioLabs inc (Table 9-5). The NEB-cloner shows which buffer is more suitable and the volumes needed for the double digestion reaction. The suggested protocol from the website (https://nebcloner.neb.com/#!/redigest) was followed.

Table 9-5. Double digestion setup reaction.

Component	50 µl reaction
DNA	1 µg
10X rCutSmart buffer	5 µl (1X)
HindIII-HF	1 µl
EcoRI-HF	1 µl
Nuclease-free water	up to 50 µl

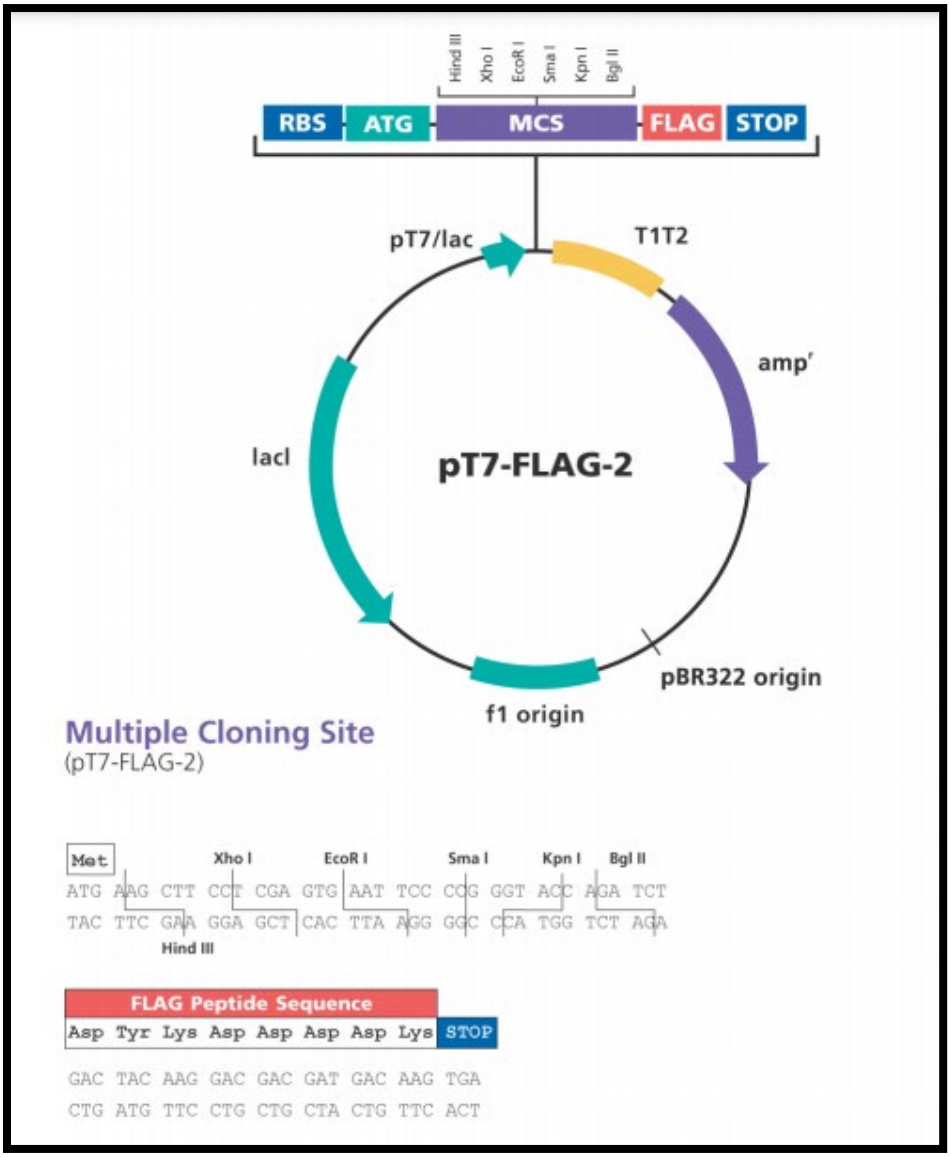


Figure 9-3. pT7-Flag-2 vector map. It is a 4815 bp *E. coli* expression vector with a multiple cloning site (MCS). It contains a FLAG epitope (DYKDDDDK) which can be detected using a monoclonal Anti-Flag M2 antibody. The T7 promoter region drives the transcription of the open reading frame (ORF) – FLAG fusion constructs. The plasmid also contains an ampicillin resistance gene. Source: (64)

9.9 Extraction of the DNAs from the agarose gel electrophoresis

The double digested products were run on agarose gel electrophoresis. The DNA fragments of the right size were cut out and extracted using the Monarch DNA Gel Extraction kit according to the instructions provided in the user manual (65). All centrifugation steps were carried out at 16 000 x g (~ 13 000 rpm). The gel parts containing the DNAs of interest were cut out. Gel dissolving buffer was added, the volume of which was determined according to the protocol where 400 µl of buffer is needed per 100 mg of agarose gel. After weighing and calculating the volume of the buffer needed, the samples were incubated at 60ºC while vortexing periodically

until the gel was completely dissolved. Samples were then loaded in the columns present inside collection tubes and centrifuged for 1 minute. Flow-through was discarded. Wash buffer (200 µl) was added, and the samples were spun down for another minute. The washing step was repeated before transferring the columns into clean 1.5 ml microfuge tubes. Approximately 20 µl of elution buffer was pipetted to the center of the matrix to elute the DNAs from the columns into the clean microfuge tubes. The DNA concentrations were measured using nanodrop.

9.10 Ligation and transformation of plasmid into DH5α cells

The ligation reaction was calculated using the Ligation Calculator provided from BioLabs inc. (https://nebiocalculator.neb.com/#!/ligation). This is a tool that calculates the mass of the insert required for a ligation reaction. According to the DNA quantification results for the empty vector and the insert DNAs, two ratios of ligation were calculated for the reaction which were 3:1 and 5:1. Based on the length of the insert DNA and the length and mass of the prokaryotic DNA vector, the tool calculates the amount of the insert DNA required for the ligation reaction. T4 buffer (ThermoFisher) (2 µl), T4-DNA-ligase (ThermoFisher) (1 µl) and nuclease-free water up to 20 µl were added to complete the reaction. The reaction mixes were gently pipetted up and down and micro-centrifuged briefly. The reactions were incubated at room temperature for 30 minutes. After incubation, the reactions were heat-inactivated at 65ºC for 10 minutes and placed on ice to proceed with the transformation into DH5α cells (Methods; Heading 9.1.2).

9.11 Colony PCR of the transformed DH5α cells

Colony PCR is a rapid method to verify the presence of the desired cloning construct in the transformed bacteria colonies that have grown on a media containing selective antibiotic (66). DreamTaq (ThermoFisher) is a high-sensitive polymerase that can amplify very low amounts of template DNA and give higher yields compared to the traditional Taq polymerases. It comes as a master-mix which contains the direct gel loading dye to simplify the downstream agarose gel analysis.

Several transformed colonies were picked with a loop, inoculated with 50 µl nuclease-free water and heated up to 100ºC for 10 minutes. The PCR reaction was set up using the DreamTaq polymerase master mix in a total volume of 50 µl (Table 9-6) under the thermocycling conditions presented in Table 9-7.

Table 9-6. Colony PCR setup. This reaction verifies the presence of the constructs pT7-Flag2:hSiglec 16 full and pT7-Flag-2:hSiglec16 extra in *E. coli* DH5α cells.

Components	50 µl reaction
DreamTaq Green Master Mix	25 µl
FWD primer	1 µl
RWD primer	1 µl
Template DNA	1 µl
Nuclease-free water	up to 50 µl

Table 9-7. Thermocycling conditions for Colony PCR.

Step	Temperature (degrees Celsius)	Time
1st Denaturation	95	3 minutes
2nd Denaturation	95	30 seconds
Annealing	67 ($hSiglec16^{full}$) | 65.7 ($hSiglec16^{extra}$)	30 seconds
Extension	72	1 minute
Final Extension	72	10 minutes
Hold	4 – 10	-

After the PCR reaction, 12 µl of each sample was directly loaded into the wells of agarose gel for visualization with electrophoresis.

9.12 Sequencing PCR for verification of the insert

Sanger sequencing is a method that reads the nucleotide bases in a DNA molecule. In addition to a DNA template and dNTPs, it needs ddNTPs (dideoxynucleotides), which are nucleotides marked with a unique fluorescent label that identifies the specific base depending on its fluorescent color. This method generates every possible length of DNA up to the full length of the target DNA to be sequenced. Every time a ddNTP is incorporated the 'end' of that DNA fragment is generated. This method is proven to be more efficient when more copies of the template DNA are added to the reaction mix (67).

The plasmids were firstly extracted and measured (Methods; Headings 9.3 and 9.4). Then the sequencing reaction was set up as shown in Table 9-8 under the thermocycling conditions of Table 9-9.

Table 9-8. Sequencing PCR setup. Sequencing can verify the proper inserted ORFs of Siglec16 full and Siglec16 extra in the pT7-Flag-2 vector.

Components	20 µl reaction
BigDye 3.1	2 µl
5X Sequencing Buffer	2 µl
DNA Template	100 – 200 ng
FWD primer | RWD primer	3.2 pM = 6.4 µl
Nuclease-free water	up to 20 µl

Table 9-9. Thermocycling conditions of sequencing PCR.

Step	Temperature (degrees Celsius)	Time
1st Denaturation	96	1 minutes
2nd Denaturation	96	10 seconds
Annealing	50	5 seconds
Extension	60	4 minutes
Hold	4 – 10	-

The sequencing PCR reaction was performed using the T7 promoter primer for pT7-Flag-2 expression vector and the corresponding Rwd primers for hSiglec16 full and hSiglec16 extra (Materials; Table 8-3). The advantage of using the T7 promoter primer is that it anneals to the pT7-Flag-2 vector upstream of its multiple cloning site (MCS), which is upstream of the GOI insert. The reaction was ran on two different clones for each of the constructed vectors with the corresponding GOI insert.

9.13 Protein Expression Studies of Siglec16 full length and Siglec16 extracellular domain in BL21 Lemo cells

E. coli Lemo21 (DE3) are chemically competent cells suitable for transformation and protein expression. According to the datasheet provided by the manufacturer (New England BioLabs inc.), these cells allow for tunable expression of different clones which is achieved by varying the level of T7 lysosome (lysY – the natural inhibitor of T7 RNA polymerase). The level of lysosome is modulated by adding L-rhamnose to the expression cultures at levels from its absence to 2000 µM. The addition of L-rhamnose tunes the expression of proteins of interest. For difficult soluble proteins such as transmembrane proteins, tuning the expression level may result in more soluble, properly folded proteins. Membrane protein expression and export in *E. coli* cells often lead to inhibition of cell division if the expression is not regulated. The Lemo21

are engineered to properly express difficult targets such as membrane proteins, toxic proteins and those prone to be insoluble through regulated expression. In this regulation, IPTG is used as an inducer and L-rhamnose as an expression regulator (68).

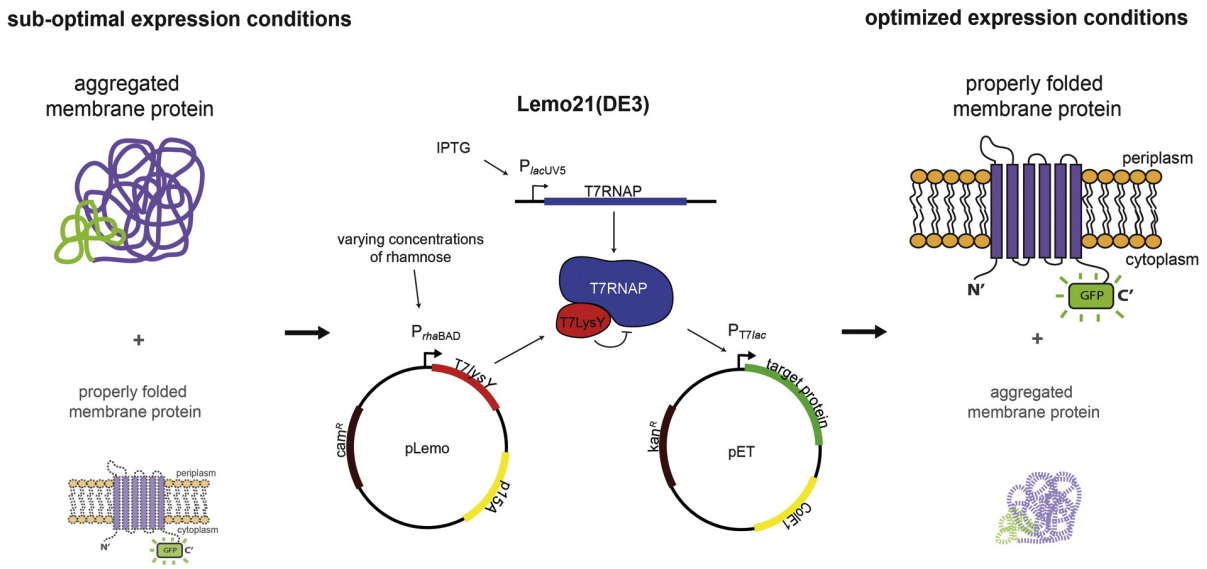


Figure 9-4. Protein expression in Lemo21 (DE3). The T7 RNA polymerase activity can be modulated by its natural inhibitor T7 lysosome (lysY). The manufacturer states that the combination of PlacUV5 expression of T7 RNA polymerase from the chromosome and L-rhamnose inducible expression of T7 lysosome from pLemo guarantees a high range of target protein expression (68). The source of the graphical abstract: (69).

A small-scale protein expression study to determine the optimal conditions for the expression of hSiglec16 full and hSiglec16 extra was performed on liquid culture using isopropyl β-D-1-thiogalactopyranoside (IPTG) as an inducer and L-rhamnose as an expression regulator. IPTG mimics a lactose metabolite (allolactose) triggering transcription of the lac operon. The manual provided by the manufacturer (70) was followed by varying the concentrations of L-rhamnose (from its absence to 750 µM). However, the IPTG inducer was kept at a constant final concentration of 400 µM. The purpose was to find the best expression conditions for the Siglec16 proteins to later on purify and use them on functional characterization studies.

9.13.1 Transformation of Lemo21 (DE3) cells with the pT7-Flag-2:hSiglec16 full and pT7-Flag-2:hSiglec16 extra plasmids

The transformation protocol (Methods; Heading 9.1.1) was followed for the transformation of the Lemo21 competent cells. The competent cells and the isolated cloned prokaryotic vectors were thawn on ice. 50 µl of competent cells were mixed with 1 µl DNA of corresponding isolated plasmids. Chloramphenicol at a concentration of 30 µg/ml was added to the selection plates which is the antibiotic that maintains the Lemo system of the competent cells. Different volumes (25 µl, 100 µl and 125 µl) for each of the constructed plasmids containing the GOIs,

were spread out onto the warmed selection plates and incubated overnight at 37ºC. A negative control which contained only competent cells was included to evaluate the presence of eventual external contaminations.

9.13.2 Protein extraction procedure from bacteria

The bacterial cells were pelleted by centrifugation at 5000 x g for 10 minutes. Lysozyme and DNase-I (2 µl per 1 ml of B-PER) were added to the B-PER reagent (ThermoFisher) to ensure efficient bacterial lysis. In addition, the same amount of 1X EDTA-free protease inhibitors were added to the mixture. The amount of B-PER needed was calculated according to the protocol which suggests that 4ml of B-PER per gram of cell pellet should be added. Based on the weight of the cell pellet which was 0.03g, 0.12 ml of B-PER reagent was added, and the suspension was pipetted up and down until homogenous. The mixtures were incubated for 30 minutes and sonicated 30 cycles on/off to achieve a homogenous mixture. Lastly, the lysates were centrifugated at 200 x g for 15 minutes at 4ºC to separate the soluble part from the insoluble part. Transferring the soluble parts into new tubes, the remaining insoluble lysates were resuspended in 200 µl filter-sterile PBS. The sonication process was an update to the instructions provided by the manufacturer (ThermoFisher).

9.13.3 Measuring of protein concentration using the Bradford Assay

Bradford assays use Coomassie dye-binding to quantify proteins. It is a fast method which can be performed in tubes or plates. These types of assays are used to specify the concentration of the proteins present in a sample.

The Coomassie Plus (Bradford) kit (ThermoFisher) was used on the protein samples extracted from the Lemo21 cells transformed with each of the constructed vectors of pT7-Flag-2:hSiglec16 full or pT7-Flag-2:hSiglec16 extra. The standard microplate protocol (provided by the manufacturer) was followed during the assay (71).

The diluted albumin (BSA) standards were prepared according to the scheme (Table 1) provided in the manual. This was done by mixing a certain amount of the diluent with a certain amount of BSA source to construct the final BSA concentration from 2000 to 0 µg/ml (blank).

The standard and samples (with unknown protein concentrations) were pipetted into a microplate and 300µl of the Coomassie Plus Reagent was added to each well. The microplate was incubated for 10 minutes and the absorbance of the samples was measured at 595nm. A standard curve was built from the blanked absorbance results and the equation provided from

the curve was used to calculate the concentration of the unknown target protein samples according to their absorbance at 595nm (Appendix 6).

9.13.4 SDS-PAGE of bacterial lysis

Polyacrylamide gel electrophoresis is a method that separates proteins based on their molecular weight/size (72). Sodium dodecyl sulfate and the polyacrylamide gel reduce the influence of the structure or charge of the proteins separating them only according to their size. The presence of SDS and a reducing agent cleaves disulfide bonds responsible for the folding of the proteins. Thus, the proteins can migrate from the cathode to the anode in its linear chain proportional to its polypeptide-chain length. Smaller protein fragments will migrate faster than the larger ones appearing in the bottom of the gel. Proteins are visualized in downstream analysis such as Coomassie staining or Western Blot.

The gel run was performed as follows. First, the 1x NuPAGE running buffer was prepared by diluting 1ml 20x stock solution in 19ml of dH_20. A precast NuPAGE (4-12%) Bis-Tris gel (Invitrogen) was assembled in the compartment for the gel electrophoresis. The running buffer was poured in the compartment, first in the inner gel space making sure it covered the entire gel without leakage. Then, the outer compartment was filled with the running buffer. The gel comb was removed in the presence of the buffer.

Prior to loading the samples into the wells, they were washed using the running buffer and a pipette. A volume of 20µl of the samples was added to each well. 4µl of the ladders SeeBlue (Invitrogen) and either BenchMark (ThermoFisher) for Coomassie staining analyses or Magic Marker (ThermoFisher) for Western Blot analyses, were loaded in other wells to determine the protein sizes. The component was placed on ice before powering the machine. The samples were run at 100V for 10 minutes allowing the loaded samples move into the gel and then between 170-200V for 1 hour.

9.13.4.1 Coomassie Staining of SDS-PAGE gels

Coomassie staining procedure was used to visualize the protein bands after SDS-PAGE electrophoresis on the diluted protein samples. It was based on the results obtained from the Bradford assay as described previously.

The polyacrylamide gel was first placed in a fixation solution (mix of 50% methanol and 10% acetic acid). The gel was covered in the fixation solution and incubated for 1 hour at RT with careful shaking. After 1 hour, the fixation solution was removed and replaced with the

Coomassie blue solution (mix of 50% methanol, 10% acetic acid and 0.05% Coomassie brilliant blue R-250). The gel was incubated for 30 minutes at RT with careful shaking. After the dictated incubation time the gel was rinsed with fixation solution once. Lastly, the de-staining solution was added (mix of 5% methanol and 7% acetic acid) for another 30 minutes at RT. The solution was changed regularly, and pieces of paper were added to improve the de-staining of the gel. A picture of the gel was taken after the last de-staining stage.

9.13.4.2 Western Blot

Western Blot is used for the detection of target proteins from a mixture of different proteins. This method can also evaluate the size and amount of the protein of interest.

15µl from the extracted protein samples were transferred in new tubes. 6µl loading dye and 2µl of reducing agent were added to all the tubes. The samples were heated for denaturation at 70ºC for 10 minutes. Prior to running the samples in the gel, they were spun down briefly to remove the condensed liquid gathered on the Eppendorf caps.

The assembly and the run of the gel was performed as described in Heading 9.13.4. Blotting was performed as follows: The nitrocellulose membrane (SigmaAldrich) was soaked for 3 seconds in methanol under the hood, then 10 seconds in water and more than 5 minutes in the blotting buffer. Pads and filter papers were also soaked in the blotting buffer for around 5 minutes. The assembly was performed as the following pattern:

table – 2 pads – filter paper – gel – membrane – filter paper – 3 pads

During the assembly, tightness of the pattern was insured as well as avoiding the creation of air bubbles. In the machine compartment blotting buffer was added on the inside and green tap water on the outside. The program NuPAGE Blot was selected in the machine, which was scheduled to run for 1 hour at 30V.

After blotting, the membrane was removed carefully and incubated for an hour in 20ml blocking buffer (5% skimmed milk in 0.05% PBSTween) at RT with slow shake. This step was performed to prevent the occurrence of non-specific binding of the antibodies. After blocking, the membrane was placed in a 50ml Falcon tube. A dilution of 1:1000 of the primary antibody in blocking buffer was prepared and added to the tube. The tube was then rotated ON at 4ºC.

The primary antibody was removed, and the membrane was washed 3x with 10 – 12 ml PBSTween (ThermoFisher) and incubated at RT for 2 minutes while rotating. A dilution of

1:1000 of the secondary antibody in blocking buffer was prepared and added to the tube. The membrane was incubated with the secondary antibody for an hour at RT while rotating. Then the membrane was washed twice for 5 minutes with 12ml PBSTween and twice for 5 minutes with 12ml washing buffer. All the washing steps were performed in rotation. The membranes were wrapped in a plastic bag in the presence of horseradish peroxidase (HRP) substrate (SigmaAldrich). HRP enzyme was used for signal amplification as it can detect the target protein by binding not directly to it but rather the antibody bound to the target.

The LumiAnalyst machine was used to scan the membranes and verify the presence, size, and amount of the target protein. The program was updated with 1 minute interval time and high sensitivity.

9.14 Gateway cloning of Siglec11 and Siglec16 in eukaryotic expression vector

The Gateway cloning is based on recombination system that is used by the lambda phage in *E. coli* to integrate its DNA in the bacterial chromosome. This occurs in the recombination sites present in the phage and in *E. coli*. It is a technology highly used for its rapid and highly efficient transfer of DNA into multiple vectors for protein expression and functional studies, as it maintains the orientation and reading frame (73).

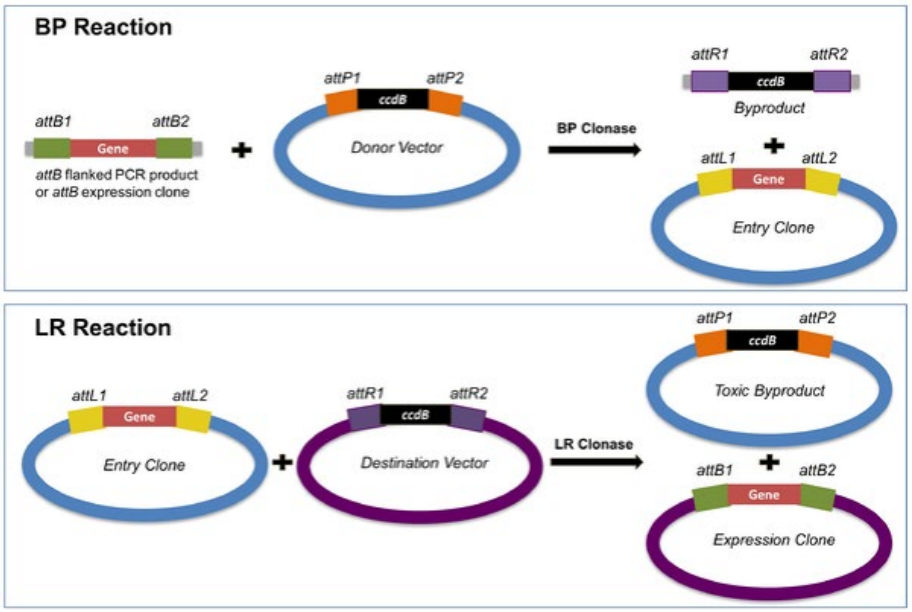


Figure 9-5. Gateway cloning system. The BP reaction happens at attB sites where it flanks the insert and attP sites of the donor vector. BP Clonase enzyme mix is used for the reaction, generating an entry clone that contains the insert DNA (attL-flanked). The LR reaction happens between attL sites of the entry clone and the attR sites of the destination vector. LR Clonase enzyme mix catalyzes the reaction, generating an expression clone that contains the insert DNA (attB-flanked). In both reactions the *ccdB* gene is excised (from the entry and expression clones). The presence of this gene in donor and destination vectors is a form

of control for the gateway cloning as the CcdD protein can inhibit the growth of several *E. coli* strains. Source: addgene.org

9.14.1 Temperature Gradient PCR and Optimal Temperature PCR for Siglec11 and Siglec16 using the attB primers

The PCR reactions for Siglec11 and Siglec16 using the attB primers were performed as described in the Methods, Heading 9.5 (Subheadings 9.5.1 and 9.5.2).

9.14.2 BP Reaction

The first step in performing the Gateway cloning is the BP reaction (BP Clonase II Mix kit from ThermoFisher) which creates an entry clone that can be used in different expression vectors depending on the aim of the study. The entry clone was created by mixing the attB PCR products and the pDONR221 plasmid (the donor vector) in a 1.5 ml tube at room temperature. 150 ng of attB-PCR product, 1 µl donor vector (150 ng/µl) and TE buffer up to 8 µl were mixed together. The BP Clonase II enzyme mix was thawed on ice for about 2 minutes, vortexed briefly (2x) and added to the reaction tube (2 µl). The tube was vortexed and micro-centrifuged 2x and incubated for an hour at 25ºC. 1 µl of Proteinase K solution was added to the reaction tube to terminate it and incubated for 10 minutes at 37º.

The BP reaction mix (1 µl) was transformed in 50 µl DH5α competent cells following the transformation protocol as in Heading 9.1.1 and plated out on LB plates containing 50 µg/ml kanamycin as the selection antibiotic for the donor vector pDONR221.

9.14.3 LR Reaction

LR reaction is the transferring reaction of GOI from an entry clone constructed in the BP reaction into a destination vector (pDEST YFP). In a 1.5 ml tube, the entry clone (150 ng), destination vector (150 ng/µl) and TE buffer (up to 8 µl) were mixed together. The LR Clonase II enzyme mix (ThermoFisher) was thawed on ice and vortexed briefly for about 2 minutes. 2 µl of the enzyme mix was added to the reaction tube and vortexed briefly. The reaction was incubated for an hour at 25ºC. 1 µl of Proteinase K solution was added to the reaction tube to terminate it and incubated at 37ºC for 10 minutes.

The LR reaction mix (1 µl) was transformed in 50 µl DH5α competent cells following the transformation protocol as in Heading 9.1.1 and plated out on LB plates containing 20 µg/ml gentamycin as the selection antibiotic for the destination vector pDEST YFP.

9.15 Infection studies in eukaryotic cells

The studies were conducted using two different cell lines: HEK293-TLR2 Blue and THP1.

HEK293-TLR2 Blue (Invivogen) were obtained by the co-transformation of the human *TLR2* (Toll-like Receptor 2) and *SEAP* (secreted embryonic alkaline phosphatase) genes into HEK293 cells which are known as Human Embryonic Kidney cells. They are known to have several advantages such as easy to transfect, rapid growth and able to produce high amounts of recombinant proteins. This cell line is usually used in protein production and interaction research (74).

THP1 are an immortalized monocyte-like cell line that were derived from the peripheral blood of a childhood case of acute monocytic leukemia. They have been used in research studies about the structure and functions of monocytes in health and disease (75).

THP1 are suitable for the study of monocyte and macrophage functions and mechanisms as they can show potential immunological response that can occur in vivo. This cell line is also used in signaling pathways and drug transport research studies (76).

9.15.1 Infection Studies in HEK293-TLR2 Blue cells transfected with either pDEST YFP:Siglec11 or pDEST YFP:Siglec16 constructs

9.15.1.1 Poly L-Lysine coating of 24 well plates

Poly L-Lysine coated (PLL) 24 well plates were used to facilitate the efficient attachment of the HEK293-TLR2 cells to the wells of the plate. They were prepared a day before seeding out the cells. 3.8 µg of PLL (~ 2µg/cm^3) was diluted in 250 µl of ultra-pure water and added to each well. The plates were then incubated overnight at 37ºC 5% CO_2. Next day, the mixture was aspired from the wells, which were then washed twice with water.

9.15.1.2 Seeding of HEK293-TLR2 cells

The number of cells to be seeded, depends on the study. The infection study was planned to be done in four different plates: A – Adhesion, B – Internalization, C – Survival and D – Transfection Efficiency according to the graphical abstracts. The wells marked Sig16 contain cells transfected with the pDEST YFP:Siglec16 construct; wells marked Sig11 are cells transfected with pDEST YFP:Siglec11 construct; the EGFP marked wells contain cells transfected with the pEGFP-C2 (Clontech) plasmid and the Non-Transf. wells represent the non-transfected cells.

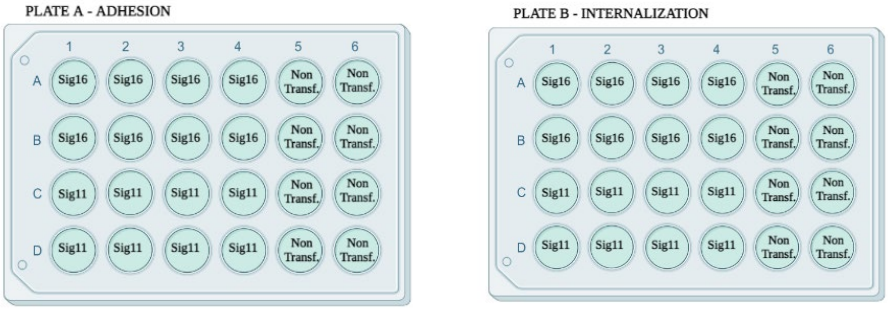


Figure 9-6. Graphical abstract of 24 well plates for adhesion and internalization assays on HEK293-TLR2.

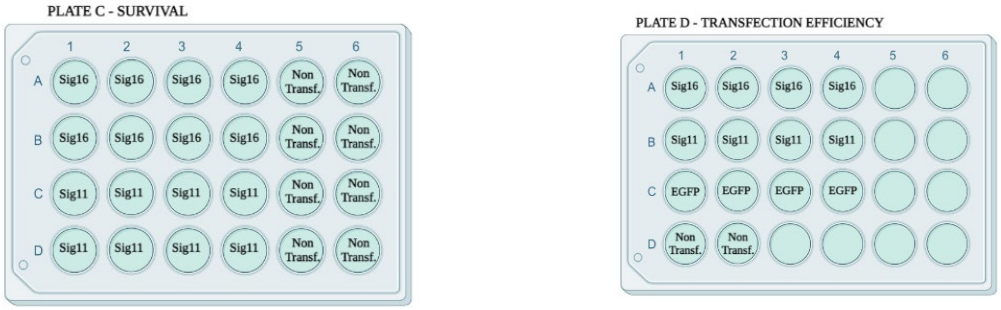


Figure 9-7. Graphical abstract of 24 well plates for survival and transfection efficiency assay on HEK293-TLR2.

Cell growth culture medium was aspirated from the culture flask. Thereafter, cells were washed twice with 10ml of sterile 1X PBS. They were detached by adding 2ml of 1X Trypsin-EDTA and incubated for 2-5 minutes at 37ºC 5% CO_2. After detachment, cells were resuspended in DMEM High Glucose (Sigma) with 10% FBS media (Sigma). The resuspended cells were seeded into PLL coated 24 well plates at a concentration of 5 x 10^5 cells/well and incubated at 37ºC 5% CO_2 for 24 hours.

9.15.1.3 Transfection of the cells with Metafectene Pro

The transfection of the cells was made using the highly efficient reagent Metafectene Pro transfection reagent (Biontex). The instructions provided by the manufacturer were followed during the transfection procedure. The cells were transfected with 500ng of the corresponding cloned vector of pDEST YFP:hSiglec11 or pDEST YFP:hSiglec16. To verify the transfection efficiency some wells were transfected with the pEGFP-C2 plasmid. After transfection, the cells were incubated for 24 hours at 37ºC 5% CO_2.

9.15.1.4 Verifying the transfection efficiency and infecting the transfected cells with *S. aureus*

Verification of the transfection efficiency was estimated using the pEGFP-C2 transfected cells in the corresponding wells on Plate D. The fluorescence microscope Leica DMI6000B was used to check the efficiency of the transfection based on the GFP green fluorescence. The cells were photographed with the 10x objective, TL-BF contrast method and with/without the GFP filter. In the other plates (A, B and C) the media was replaced with fresh warm DMEM High Glucose + 10% FBS media and placed back in the incubator after ensuring that the cells were healthy.

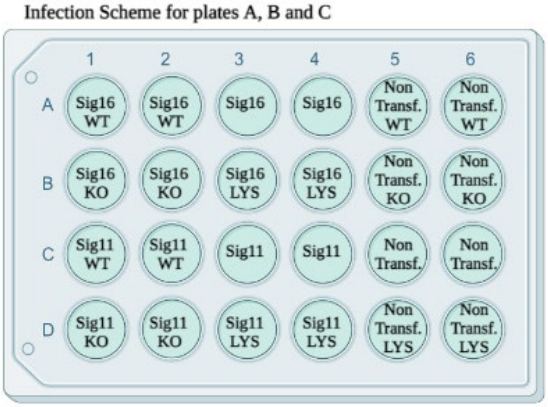
9.15.1.5 Bacterial preparation and infecting the transfecting cells with *S. aureus*

Same day the mammalian cells were transfected, overnight cultures (ON) of *S. aureus* NCTC8325-4 (Wild-Type) and NCTC8325-4Δs*drD* (Knock-Out) were prepared in 5ml TSB incubated at 37ºC with shaking at 220 rpm.

200 µl from the *S. aureus* ON cultures was added to 10 ml fresh TSB and incubated for 2 hours until they reached an OD_{600} of 0.920 A for the NCTC8325-4 and 0.795 A for the NCTC8325-4Δ*sdrD*. The bacterial cultures were then centrifuged at 5000 rpm at RT for 10 minutes. 50 ml of PBS was used to wash the cultures twice before resuspending them in 1 ml DMEM High Glucose + 10% FBS. 10 ml of fresh DMEM media was mixed respectively with 590 µl of the NCTC8325-4 bacteria and 850 µl of the washed NCTC8325-4Δ*sdrD* bacteria to constitute the OD_{600} = 0.4 for each of them which usually indicates that the number of bacteria is approximately 10^8 cells/ml.

The HEK293-TLR2 cells were recounted after two days (1.8 x 10^5 cells/well). This cell number was used to prepare the bacterial suspensions giving a multiplicity of infection (MOI) of 1. The old media was aspirated from the wells in plates A, B and C. 0.5 ml of infection medium was

added accordingly based on the Infection Scheme below. Fresh media was added to the non-infected cells.



After infection the plates were incubated for 90 minutes at 37ºC 5% CO_2.

- At 90 minutes, 500 µl of media containing antibiotics (20 µg/ml lysostaphin and 2mg/ml floxacillin) was added to all the wells in plates B and C.
- Plate B was incubated for an additional hour.
- Plate C was incubated overnight

9.15.1.6 Treatment of the plates according to their respective time points

15 minutes before the end of incubation, 10 µl of lysis buffer 10x (kit by Promega) was added to the LYS wells and incubated for the remaining time. This was the positive control used further on for the cell cytotoxicity assay.

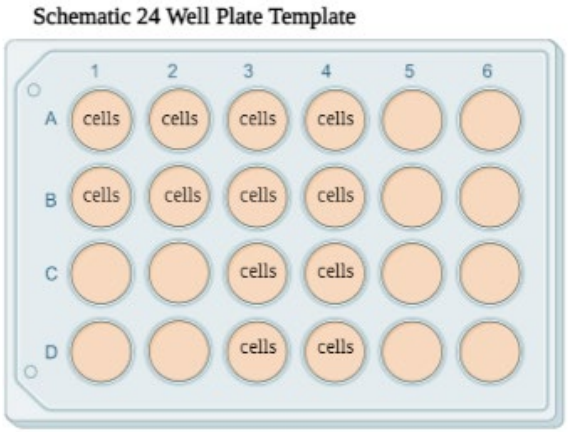
Supernatants were harvested from all wells, (supernatants from the two technical replicates were combined in one tube) and stored at – 20ºC. The remaining cells were then washed gently twice with PBS. The PBS was applied to the side of the wells to ensure that the washing did not dislodge the cells. The plates were incubated for 5 minutes with 100 µl of trypsin EDTA in all of the wells. To lyse the mammalian cells, 400 µl of filter sterile PBS + 1% Triton X-100 was added. To make sure the cells were properly lysed, the supernatant was pipetted up and down several times. The supernatants were collected. The diluted supernatants were plated out on TSA plates and incubated at 37ºC ON. Bacteria colonies were counted the next day.

9.15.2 Infection Studies in THP1 cells with and without recombinant Siglec11

To study the biological function of Siglec11 when cells are infected with *S. aureus* strains, a second strategy was used. This involved the differentiation of THP1 cells into macrophages and adding of commercially purchased recombinant protein of Siglec11 (RnD USA) during the bacterial infection.

9.15.2.1 Seeding of THP1 cells

The THP1 cell suspension was harvested, washed once with PBS, and resuspended in RPMI (Sigma) + 10% FBS media. The cells were counted using Scepter™ 2.0 cell counter. Cell suspension of 5 x 10^5 cells/ml was prepared in RPMI + 10% FBS media. 20ng/ml of Phorbol 12-myristate 13-actetare (PMA) was added and mixed gently to stimulate the differentiation of THP1 cells. 0.5 ml from the suspension was added to each well in 24 well plates based on the following template:



The plates were incubated for 48 hours at 37ºC 5% CO_2. After 48 hours, the THP1 cells were fully adherent and differentiating. The media was replaced with warm RPMI + 10% FBS and placed back in the incubator.

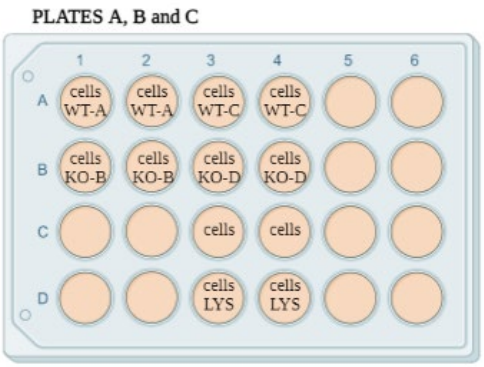
9.15.2.2 Infection of THP1 cells with *S. aureus*

24 hours prior to infection, ON cultures of *S. aureus* NCTC8325-4 (Wild-Type) and NCTC8325-4Δ*sdrD* (Knock-out) were prepared in 5ml TSB incubated at 37ºC with shaking at 220 rpm.

0.2 ml of ON cultures of *S. aureus* NCTC8325-4 and NCTC8325-4Δ*sdrD* was added into 10 ml fresh TSB and incubated for 2 hours. After 2 hours, the OD_{600} for the NCTC8325-4 was 1.025 A and for the NCTC8325-4Δ*sdrD* 0.844 A. The bacterial cultures were centrifuged at 5000 rpm at RT for 10 minutes and washed twice with PBS. The bacteria were resuspended in 1ml RPMI + 10% FBS media and each of the strains were diluted to OD_{600} = 0.4. 4 different infection mediums were prepared giving the infection of MOI = 5:

A. 8 ml of *S. aureus* NCTC8325-4 suspension in RPMI + 10% FBS media
B. 8 ml of *S. aureus* NCTC8325-4Δ*sdrD* suspension in RPMI + 10% FBS media
C. 8 ml of *S. aureus* NCTC8325-4 suspension in RPMI + 10% FBS media and 1 µg/ml of recombinant protein Siglec11
D. 8 ml of *S. aureus* NCTC8325-4Δ*sdrD* suspension in RPMI + 10% FBS media and 1 µg/ml of recombinant protein Siglec11

The volume of bacterial suspension needed was 0.2 ml. The recombinant protein Siglec11 was reconstituted in a 100 µg/ml volume and 0.08 ml was added in the C and D suspensions. The cells were infected in the following pattern in all of the plates where letters A, B, C and D represent the bacterial suspensions:



After infecting the cells, the plates were centrifuged at 500 x g at RT for 5 minutes and incubated for 30 minutes at 37ºC 5% CO_2. After incubation, RPMI media containing antibiotics (20 µg/ml lysostaphin and 2 mg/ml floxacillin) was added to all the wells. The plates were incubated at 37ºC 5% CO_2:

- Plate A for an additional 2 hours

- Plate B for an additional 5 hours
- Plate C overnight

Treatment of the plates was performed as in Heading 9.15.1.6.

9.16 Cytotoxicity assay on the harvested supernatants

The CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega) was used according to the manual provided by the manufacturer (77). The assay measures lactate dehydrogenase (LDH) which is a stable cytosolic enzyme that is released upon cell lysis. It is measured in supernatants harvested from the cell cultures with a 30-minutes coupled enzymatic assay, which results in the conversion of a tetrazolium salt into a red product. The amount of colored formed is proportional to the number of lysed cells. This assay can reveal early, low-level cytotoxicity. Visible wavelength absorbance data are collected using a standard 96-well plate reader. 50 µl of supernatant from eukaryotic cells were mixed in duplicates with 50 µl of CytoTox reagent pre-prepped by adding 12 ml of assay buffer in a substrate mix bottle. The plate was incubated for 30 minutes at RT after adding the CytoTox reagent. The plate was covered with foil after adding the reagent to protect it from light. After incubation, 50 µl of Stop solution was added to each well and the absorbance was recorded at a wavelength of 490 nm within one hour.

9.17 Human IL-6 ELISA assay

The Human IL-6 ELISA kit (Thermofisher) was used to measure the amount of the target (Interleukin-6) bound between a matched antibody pair. An antibody specific to the target is pre-coated in the wells of a 96-well plate. The standards, samples and the controls were added in duplicates in the wells to bind to the immobilized antibody. The sandwich was formed when an additional secondary antibody was added to the mix which reacts with the enzyme-antibody-target complex to produce a measurable signal. The intensity of this signal is directly proportional to the concentration of IL-6 that is present in the supernatants harvested from the cell cultures. The manual provided by the manufacturer was followed for this assay (78). All reagents were in RT before use. Dilution of the washing buffer, the standards, and the preparation of the secondary antibody (Streptavidin-HRP 100X) were prepared prior to running the assay according to the instructions in the manual. The standard curve and all the samples were made in duplicate, and the plate was measured at OD_{450} nm. The total incubation time was 4 hours.

10 Results

At the beginning of the thesis, a commercial antibody for the detection of Siglec16 was unavailable for purchase. Therefore, its DNA was cloned into a Flag-tagged prokaryotic vector, to ensure the expression of recombinant hSiglec16 full length and hSiglec16 extracellular domain in *E. coli* DH5α cells. The cloning procedure resulted in the following constructs:

1. pT7-Flag-2:hSiglec16 full – a prokaryotic expression vector containing the ORF for the full length of human Siglec16 protein
2. pT7-Flag-2:hSiglec16 extra – a prokaryotic expression vector containing the ORF for the extracellular domain of human Siglec16 protein

10.1 High quality plasmid DNA obtained from transformed DH5α

Plasmid DNA concentrations isolated from the transformed *E. coli* cells are presented in Table 10-1. Two clones were chosen for each of the plasmids. Clones A and B of the pT7-Flag-2 had a concentration of 240.6 and 211.3 ng/µl respectively. Clones A and B of phSiglec16 had higher concentrations of 690.3 and 642.6 ng/µl respectively. The $A_{260nm}/_{280nm}$ ratio for all DNAs were between 1.7 to 1.87.

Table 10-1. Concentration of plasmid DNAs isolated from DH5α. The unit of measurement is ng/µl. The ratio of the absorbance at 260 and 280 nm addresses the purity of the DNAs.

Sample ID Nucleic Acid	Concentration	Unit	$A_{260-280}$
pT7-Flag-2 Clone A	240.6	ng/µl	1.70
pT7-Flag-2 Clone B	211.3	ng/µl	1.71
phSiglec16 Clone A	690.3	ng/µl	1.88
phSiglec16 Clone B	642.6	ng/µl	1.87

10.2 Optimal annealing temperature for the amplification of hSiglec16 full and hSiglec16 extra determined from the respective gradient PCRs.

Images of agarose gel, Figures 10-1 and 10-2, visualize the amplification of hSiglec16 full and hSiglec16 extra fragments. Samples S1-S6 for the full length (Figure 10-1) and S1-S4 for the extracellular domain (Figure 10-2) represent the tested annealing temperatures. Controls were included to verify the success or failure of the reaction. C1 is a control in the absence of template DNA while C2 is a control in the absence of the Phusion polymerase.

All samples tested for both DNA fragments, show the expected band sizes of 1458 bp for hSiglec16 full and 1194 bp for hSiglec16 extra. However, based on the integrity of the bands, samples S2 were chosen as the optimal annealing temperatures for the DNA fragments of interest: 67ºC for hSiglec16 full and 65.7ºC for hSiglec16 extra. Further analyzing the gel images, the absence of bands in the control samples (C1 and C2) support the overall success and specificity of the reactions.

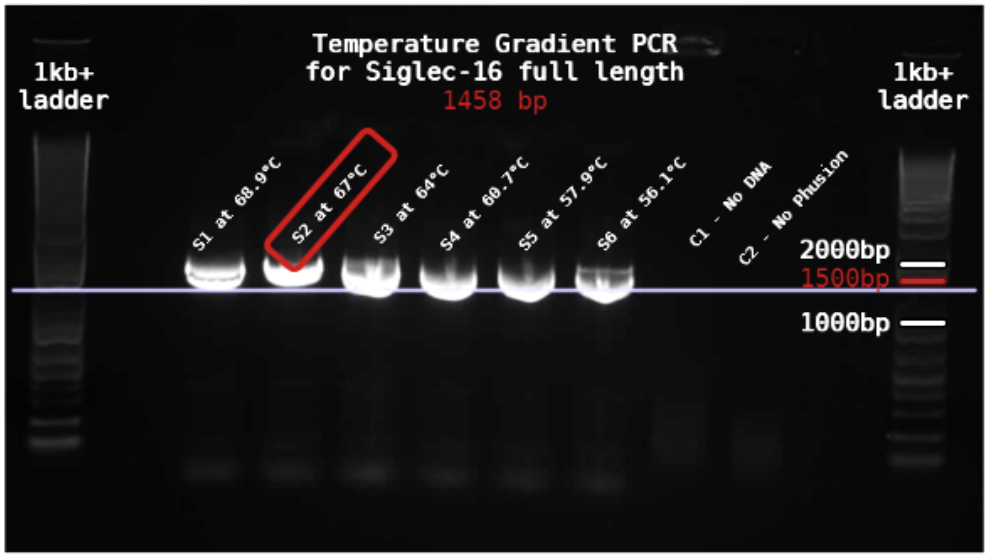


Figure 10-1. Agarose gel image of gradient PCR for hSiglec16 full fragment. PCR reactions were tested at several annealing temperatures to determine the optimal one for the amplification of the full hSiglec16 fragment and were ran on 1% agarose gel; the 1kb+ DNA molecular weight ladder with the indicated 1kb, 1.5kb, and 2kb bands is labeled on the image; S1-S6 samples are the amplified DNA full fragments at specific annealing temperatures; their band sizes seem all under the 1.5 kb ladder band size; C1 and C2 are the control samples in the absence of template and polymerase respectively.

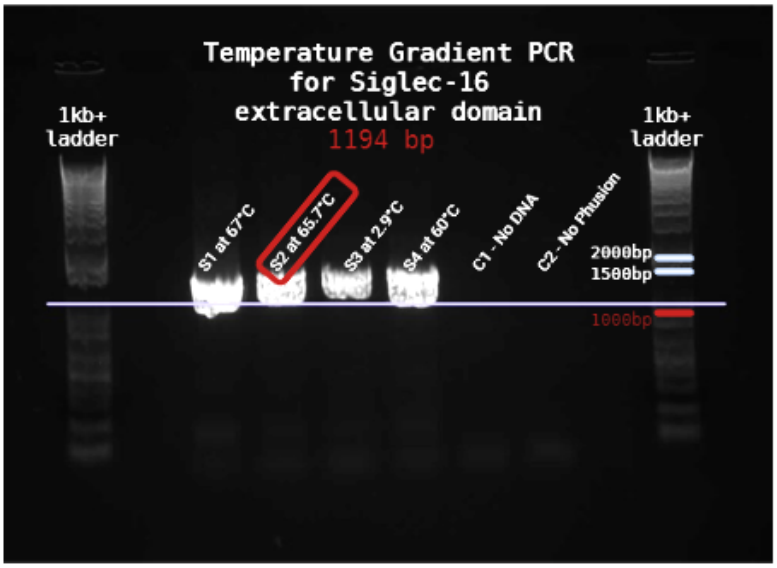


Figure 10-2. Agarose gel image of gradient PCR for hSiglec16 extra fragment. PCR reactions were tested at several annealing temperatures to determine the optimal one for the amplification of the extracellular domain hSiglec16 fragment and were ran on 1% agarose gel; the 1kb+ DNA molecular weight ladder with the indicated 1kb, 1.5kb, and 2kb bands is labeled on the image; S1-S4 samples are the amplified DNA extra fragments at specific annealing temperatures; their band sizes seem all over the 1 kb ladder band size; C1 and C2 are the control samples in the absence of template and polymerase respectively.

Based on the S2 sample temperatures for each of the fragments, an optimal PCR was performed for their additional amplification. Figure 10-3 is the gel image of the reaction where the amplification of both hSiglec16 fragments were verified by the expected band sizes of ~1.4 bp for hSiglec16 full and ~1.2 bp for hSiglec16 extra. Controls C1 and C2 as in the previous gel images were reincluded. Small-faded bands appear in the controls of hSiglec16 full reaction.

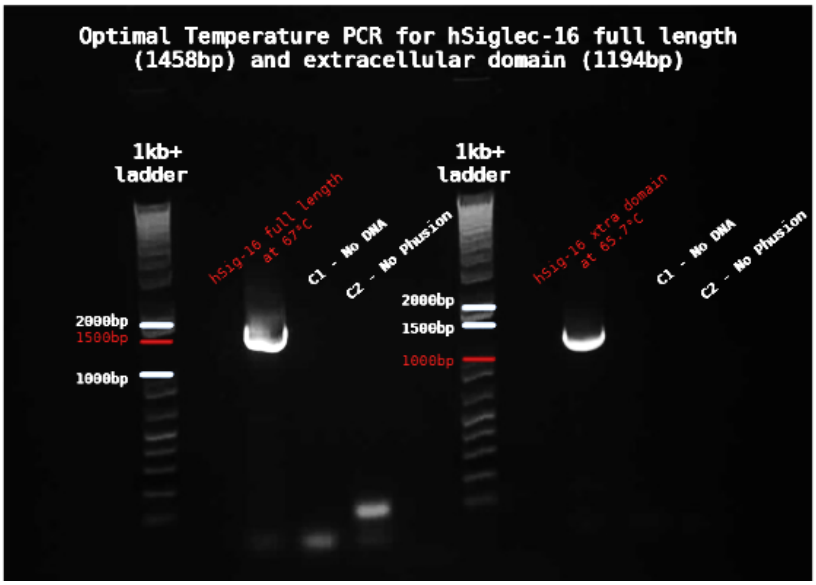


Figure 10-3. Analysis and confirmation of the amplification of hSiglec16 full and extra fragments. 1kb+ ladder is indicated in the image; the amplified fragments at their respective temperatures are marked in red over their specific bands in sizes ~1.4 bp for hSiglec16 full and ~1.1 bp for hSiglec16 extra.

10.3 DNA concentration of PCR products of hSiglec16 full and extra fragments resulted high after product cleanup

Purified PCR products of hSiglec16 full and extra fragments had a concentration of 444.6 and 312.9 ng/µl respectively. The $A_{260nm/280nm}$ ratio for all the clones were between 1.88 to 2.07.

Table 10-2. Concentration of purified PCR products of hSiglec16 full and extra DNAs. The fragments were amplified during optimal temperature PCR; the unit of measurement is ng/µl. The absorbance ratio at 260nm and 280 nm were over 1.8 verifying the purity of the nucleic acids.

Sample ID Nucleic Acid	Concentration	Unit	$A_{260-280}$
hSiglec16 full	444.6	ng/µl	2.07
hSiglec16 extra	312.9	ng/µl	1.88

10.4 Digested products of pT7-Flag-2, hSiglec16 full and hSiglec16 extra visualized in agarose gel

Restriction enzymes HindIII-HF and EcoRI-HF were used in the double digestion reaction and ran on 1% agarose gel as shown in Figure 10-4. The bands appear slightly higher in their corresponding lanes and the 1kb+ ladder appears tighter and less stretched out than expected. However, taking in consideration the high product concentrations as observed from previous results, the obtained gel image was accepted as accurate. The specificity of the bands and the absence of any contamination support the success of the digestion.

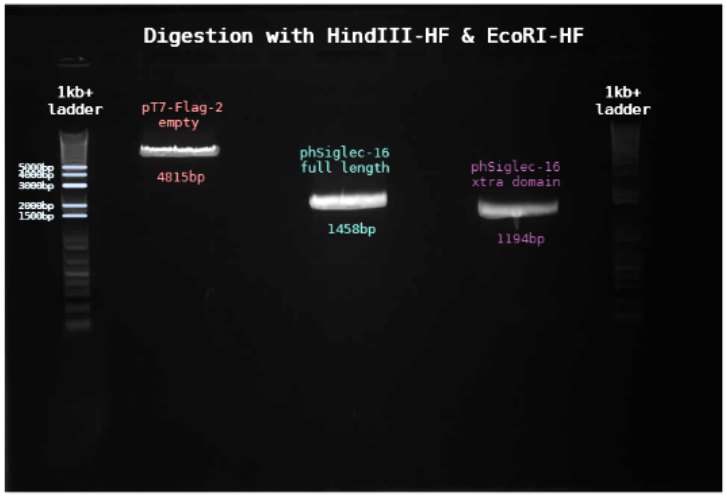


Figure 10-4. Gel image of the double-digested products. The ladder bands appear smaller and faded; the identity of the bands is marked on top while its size on the bottom; the image appears clear of any eventual contamination.

Table 10-3 presents the quantification values of the purified digested products that were extracted from the agarose gel. pT7-Flag-2 had the highest concentration of 202.1 ng/µl while the hSiglec16 fragments had lower concentrations of 9.5 (full) and 11.5 (extra) ng/µl. The absorbance ratio values vary from 1.67 to 1.77 indicating that the products are not completely pure.

Table 10-3. DNA concentrations of the purified digested products.

Sample ID Nucleic Acid	Concentration	Unit	$A_{260-280}$
pT7-Flag-2 vector	202.1	ng/µl	1.71
hSiglec16 full	9.5	ng/µl	1.67
hSiglec16 extra	11.5	ng/µl	1.77

These products were thereafter used in the ligation reaction to build the desired constructs (unpublished results).

10.5 Colony PCR confirmed the construction of pT7-Flag-2:hSiglec16 full and pT7-Flag-2:hSiglec16 extra

More colonies were observed in the ligation ratio 3:1 (insert DNA/vector DNA) agar plate comparted to the 5:1 ratio (unpublished images). Colony PCR was performed on several randomly chosen colonies to verify the presence of the GOIs. In Figure 10-5, almost all transformants that contain the pT7-Flag-2:hSiglec16 full construct appear on the expected band size of ~1.4 bp, aside for clone C3 (from the 5:1 ligation ratio). Figure 10-6 shows all the expected band sizes from the transformants containing the pT7-Flag-2:hSiglec16 extra construct.

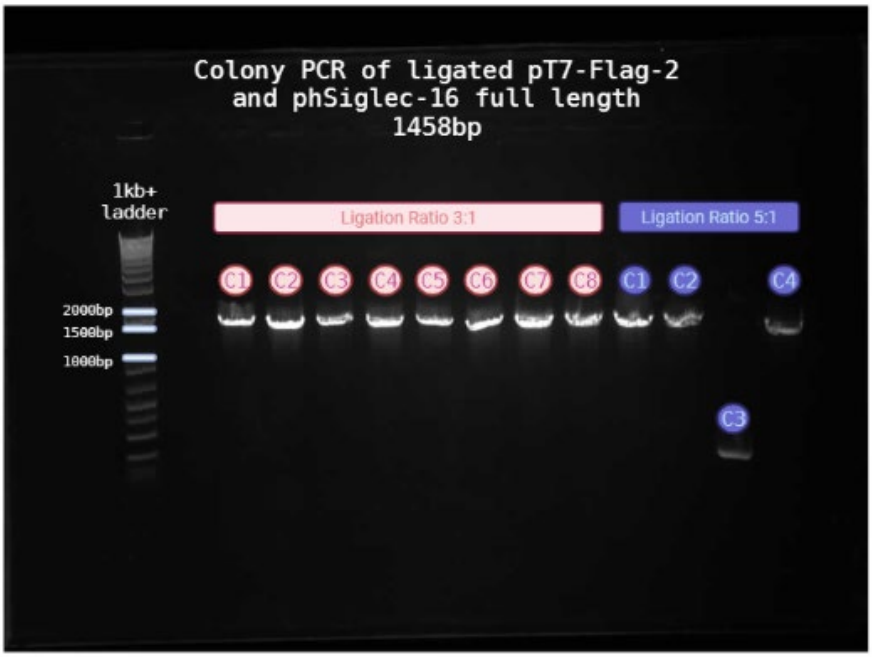


Figure 10-5. Gel image of PCR performed on colonies, transformed with pT7-Flag-2:hSiglec16 full. Several colonies were selected from two ligation ratios 3:1 and 5:1 indicated in the image in pink and blue boxes respectively; transformants are identified as C1-C8 from 3:1 ratio and C1-C4 from 5:1 ratio; the 1kb+ ladder indicates band sizes of 1kb, 1.5kb and 2kb.

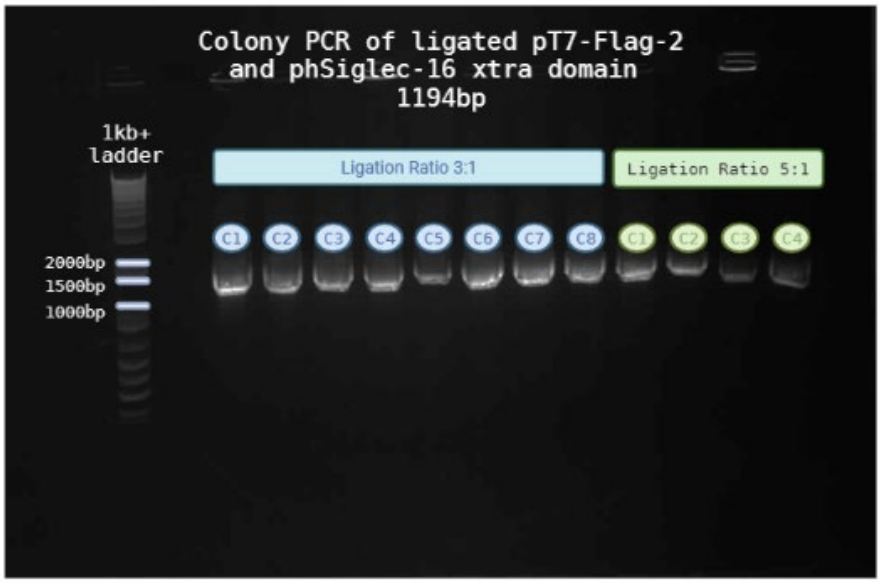


Figure 10-6. Gel image of PCR performed on colonies, transformed with pT7-Flag-2:hSiglec16 extra. Several colonies were selected from two ligation ratios 3:1 and 5:1 indicated in the image in light blue and green boxes respectively; transformants are identified as C1-C8 from 3:1 ratio and C1-C4 from 5:1 ratio; the 1kb+ ladder indicates band sizes of 1kb, 1.5kb and 2kb.

10.6 Direct sequencing verified the presence of the hSiglec16 extra sequence but not that of hSiglec16 full

Query sequences of pT7-Flag-2:hSiglec16 full, clones A and B resulted in less than 300 sequence reads, thus no hits were generated when blasted against the NCBI database (Table 10-4).

Query coverage is the percentage of the obtained query sequence from the sequencing reaction aligned to a sequence present in the database. The E-value is a parameter which shows how many hits can be expected by chance and it decreases exponentially as the match increases thus, the lower it is the more significant the match is. The identity percentage are identical residues that match the hit. Sequences that are closely related together have a higher identity percentage.

Table 10-4. Top Blast Hits for A and B clones of pT7-Flag-2:hSiglec16 full

Plasmid DNA	Primer	Top Blast Hit	Query Cover	E-value	Identity Percentage
pT7-Flag-2:hSiglec16 full Clone A	T7 promoter	-			
pT7-Flag-2:hSiglec16 full Clone A	Rwd for hSiglec16 full	-			
pT7-Flag-2:hSiglec16 full Clone B	T7 promoter	-			
pT7-Flag-2:hSiglec16 full Clone B	Rwd for hSiglec16 full	-			

However, a 252bp query sequence was obtained from pT7-Flag-2:hSiglec16 full clone B when aligned with the complete sequence of the prokaryotic expression vector pT7-Flag-2. The T7 promoter is located in the position 72-91 and the starting position of the MCS is 159-194. The alignment shown in the Figure 10-7 shows an identity of 97% between the sequences in positions 70-164 which may indicate that the GOI might be in frame in the constructed pT7-Flag-2:hSiglec16 full.

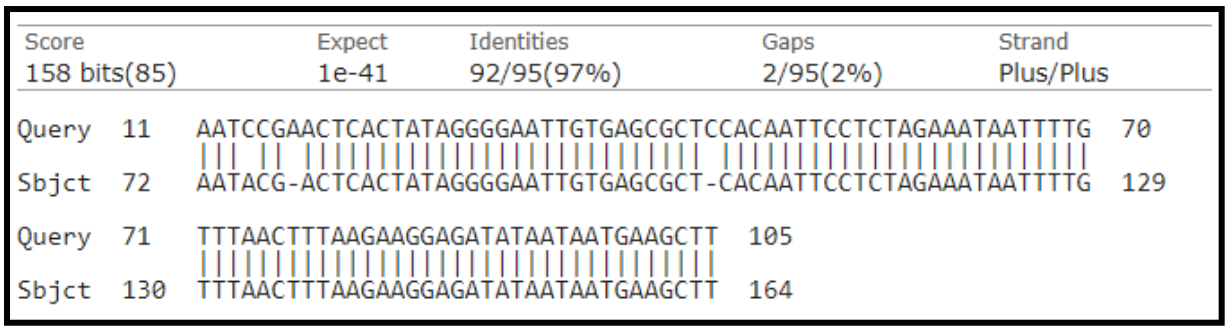


Figure 10-7. Alignment of pT7-Flag-2:hSiglec16 full query sequence against pT7-Flag-2 sequence.

Query sequences of pT7-Flag-2:hSiglec16 extra resulted in over 1000 bp reads. The obtained sequence reads were blasted against the NCBI database. The generated information is provided in Table 10-5. All the blasted query sequences indicated the presence of GOI in the constructed pT7-Flag-2:hSiglec16 extra vector. However, the position of the GOI in the vector could not be determined.

Table 10-5. Top Blast Hits for A and B clones of pT7-Flag-2:hSiglec16 extra.

Plasmid DNA	Primer	Top Blast Hit	Query Cover	E-value	Identify Percentage
pT7-Flag-2:hSiglec16extra Clone A	T7 promoter	hSiglec16 mRNA, complete cds	90%	0.0	97.40%
pT7-Flag-2:hSiglec16extra Clone A	Rwd for hSiglec16extra	hSiglec16 mRNA, complete cds	96%	0.0	95.70%
pT7-Flag-2:hSiglec16extra Clone B	T7 promoter	hSiglec16 mRNA, complete cds	89%	0.0	97%
pT7-Flag-2:hSiglec16extra Clone B	Rwd for hSiglec16extra	hSiglec16 mRNA, complete cds	91%	0.0	98.19%

10.7 Neither Siglec16 full nor hSiglec16 extra were identified in SDS-PAGE of Lemo21 lysates

Protein to DNA conversions according to genscript.com was used to calculate the expected sizes for Siglec16 full and extra proteins. 1kb DNA = 333 amino acids in a 37kDa protein size. Therefore, Siglec16 full was expected ~54kDa while Siglec16 extra ~44kDa.

Lemo21 cells were induced by IPTG at a constant concentration of 400μM. L-rhamnose was absent in some samples and at a concentration of 750μM in others. The samples were separated in two fractions: soluble (S) and insoluble (IS). The lysates appeared highly concentrated and unidentifiable (unpublished images) in first SDS-PAGE runs therefore, Bradford assay was performed (Appendix 6) and the samples were diluted to a concentration of ~1000 μg/ml.

Figure 10-8 is a gel image aimed to compare uninduced control samples. Induced samples were loaded in the gel showed in Figure 10-9. A similar protein background was noticed on almost all loaded lysate samples. Thus, the detection of the target proteins of Siglec16 full and extra were impossible to distinguish and identify.

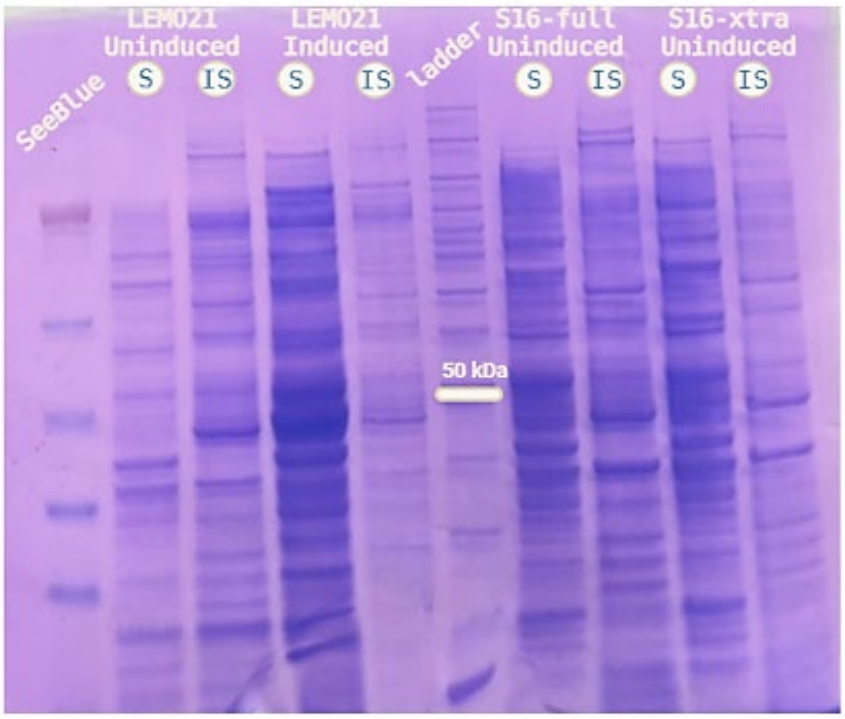


Figure 10-8. Coomassie Blue stained gel image. Uninduced samples were loaded in gel used as control for the identification of target proteins; Lemo21 induced/uninduced are the non-transfected cells; uninduced cells transformed with pT7-Flag-2:Siglec16 full and extra constructs are labeled as S16-full and S16-xtra respectively; (S) are the soluble while (IS) the insoluble fractions; SeeBlue standard and BenchMark ladder are included to identify the band sizes (50kDa band is indicated in the ladder).

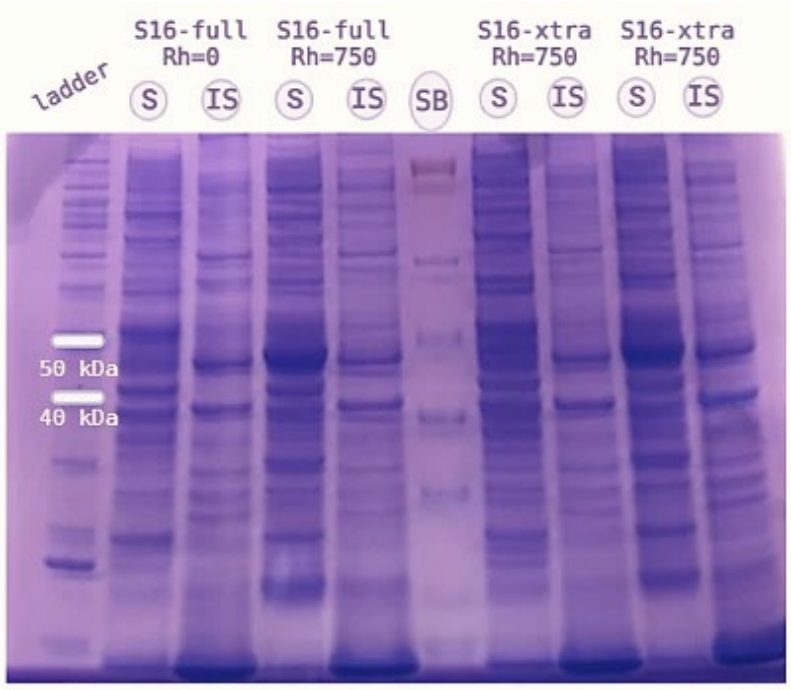


Figure 10-9. Coomassie Blue stained gel image. IPTG induced samples loaded to identify target proteins; Rh=0 are the samples in the absence of L-rhamnose while Rh=750 are samples where the concentration of the rhamnose regulator is 750μM; (S) and (IS) are the soluble and insoluble fractions of the lysates; band sizes of 40 and 50kDa are indicated in the ladder; SB is the SeeBlue loaded to visually track the migration of the samples.

10.8 The monoclonal Flag M2 antibody in Western Blot resulted unspecific and failed to detect Siglec16 full and extra

Western Blot analysis where the monoclonal Flag M2 antibody (produced in mouse) was used for the specific detection of the target proteins. The pT7-Flag-2 vector contains a Flag tag in the C-terminus. It is located after the MCS where the ORFs of the GOIs were inserted (Figure 9-3) and it can be detected by the M2 antibody. The S fractions were loaded in one gel whereas the IS fractions were loaded in another gel for easier comparison and detection.

The M2 antibody is specific to the Flag-2 tag however Figures 10-10 and 10-11 present the appearance of several bands in all loaded wells. The comparison of the control samples with the induced ones failed to identify Siglec16 full and/or extra proteins.

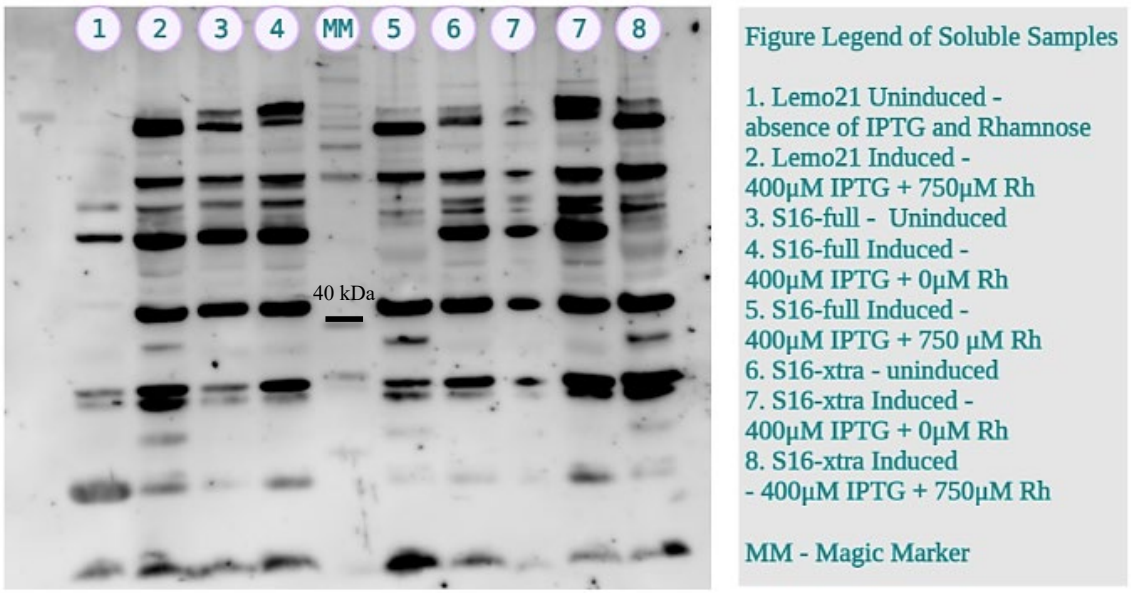


Figure 10-10. Western Blot image of the bacterial lysates. Monoclonal-Flag-M2 antibody was used for the detection of hSiglec16 full and hSiglec16 extra; the figure legend on the right identifies the samples that correspond with the numbers in the product lanes on the gel scan; Magic Marker™ XP (MM) is used to identify the protein band sizes; 40kDa band size is indicated in the ladder.

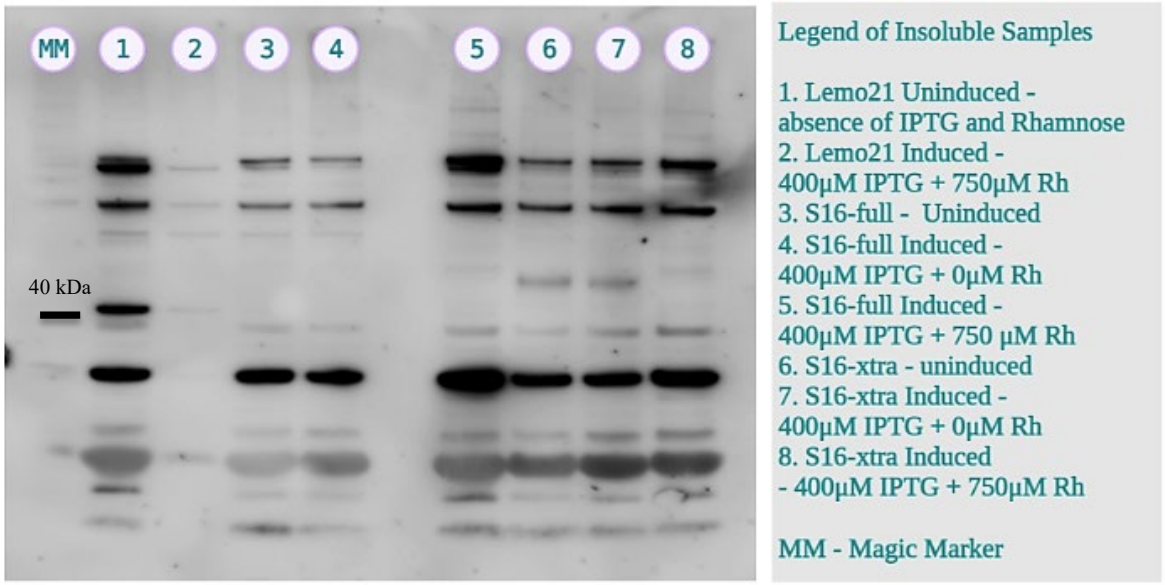


Figure 10-11. Western Blot image of the bacterial lysates. Monoclonal-Flag-M2 antibody was used for the detection of hSiglec16 full and hSiglec16 extra; the figure legend on the right identifies the samples that correspond with the numbers in the product lanes on the gel scan; Magic Marker™ (MM) was used to identify the protein band sizes; 40kDa band size is indicated in the ladder.

10.9 The human Siglec16 antibody in Western Blot analyses identified a band corresponding to the Siglec16 extra protein size

Figures 10-12 and 10-13 at a 3-hours induced bacterial growth, indicated specific bands of the Siglec16 extra protein with the expected size of ~44kDa. The antibody concentration (0.1µg/ml) was determined based on Human Siglec11 antibody due to their significant similarity of their extracellular domains. Moreover, the Human Siglec11 antibody can also detect Siglec16 protein (50).

Two bands are visible in Figure 10-12: one represents the uninduced Lemo21 (DE3) cells transformed with the pT7-Flag-2:hSiglec16 extra and the other on the right, the induced Lemo21 (DE3) with 400µM IPTG in the absence of L-rhamnose which shows an increased signal amplification. Furthermore, in this latter product lane, a fade unexpected band at a size larger than 50kDa is visible.

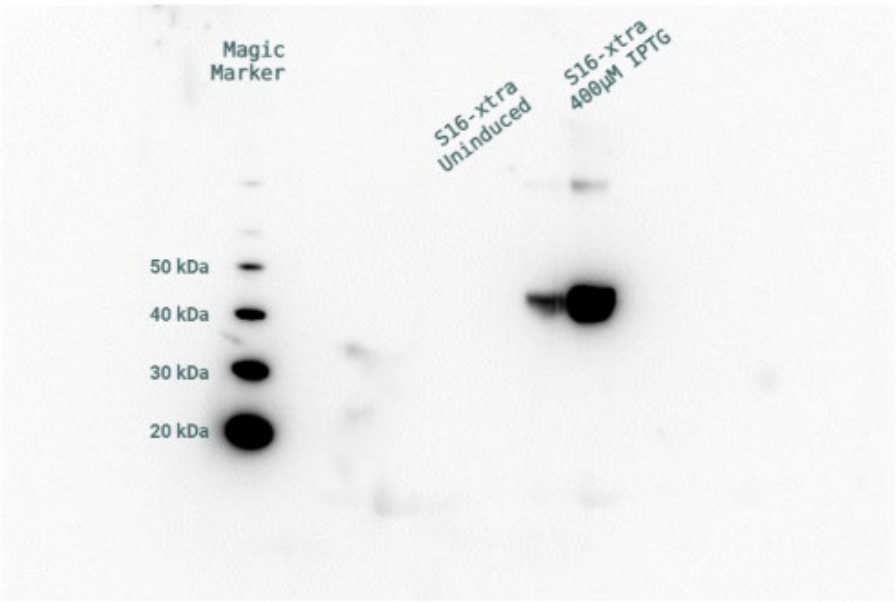


Figure 10-12. Western Blot image using the human Siglec16 antibody. It shows the soluble fractions of the extracted samples cultured for 3-hours after IPTG induction; MagicMarker™ is the protein ladder that identifies the sizes of the observed bands in the gel; sizes 20kDa to 50kDa are indicated in the figure; HRP was used for signal amplification.

In the insoluble fractions (Figure 10-13) a fade band of the induced Lemo21 (DE3) cells transformed with the pT7-Flag-2:hSiglec16 extra is visible at the expected size (~44kDa), possibly indicating traces of the expressed hSiglec16 extra target protein.

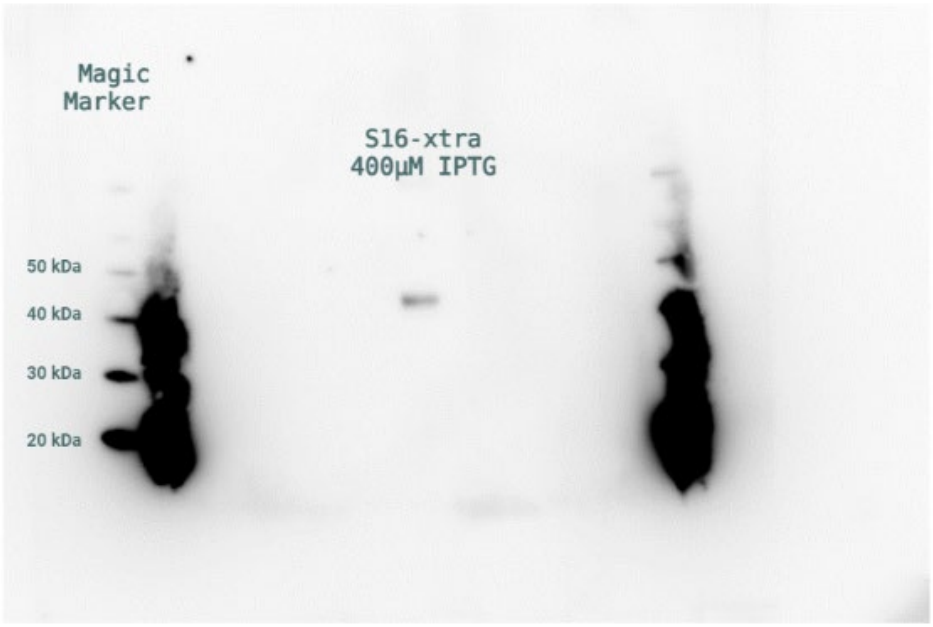


Figure 10-13. Western Blot image using human Siglec16 antibody. It shows the insoluble fractions of the extracted samples cultured for 3-hours after IPTG induction; MagicMarker™ is the protein ladder that identifies the sizes of the observed bands in the gel; sizes 20kDa to 50kDa are indicated in the figure; HRP was used for signal amplification.

10.10 Optimal annealing temperature for the amplification of hSiglec16 and hSiglec11 determined from the respective gradient PCRs.

Figures 10-14 and 10-15 show the PCR amplification of fragments of hSiglec11 and hSiglec16 when visualized by agarose gel electrophoresis. Samples S1-S6 for both fragments are amplified in different annealing temperatures. Controls C1 (absence of template DNA) and C2 (absence of Phusion polymerase) were included to determine the success or failure of the reactions.

For both fragments, most of the samples show approximately the expected band sizes of ~2.1kb for hSiglec11 and ~1.4kb for hSiglec16. However, based on band's integrity, the average temperature of S3 and S4 for hSiglec11 (Figure 10-14) was chosen as the optimal annealing temperature. For hSiglec16 fragment PCR amplification, the average of S5 and S6 (Figure 10-15) was calculated as the optimal annealing temperature. Furthermore, several bands were noticed in the controls included in both PCR amplifications of hSiglec11 and hSiglec16 fragments (Figures 10-14 and 10-15 respectively).

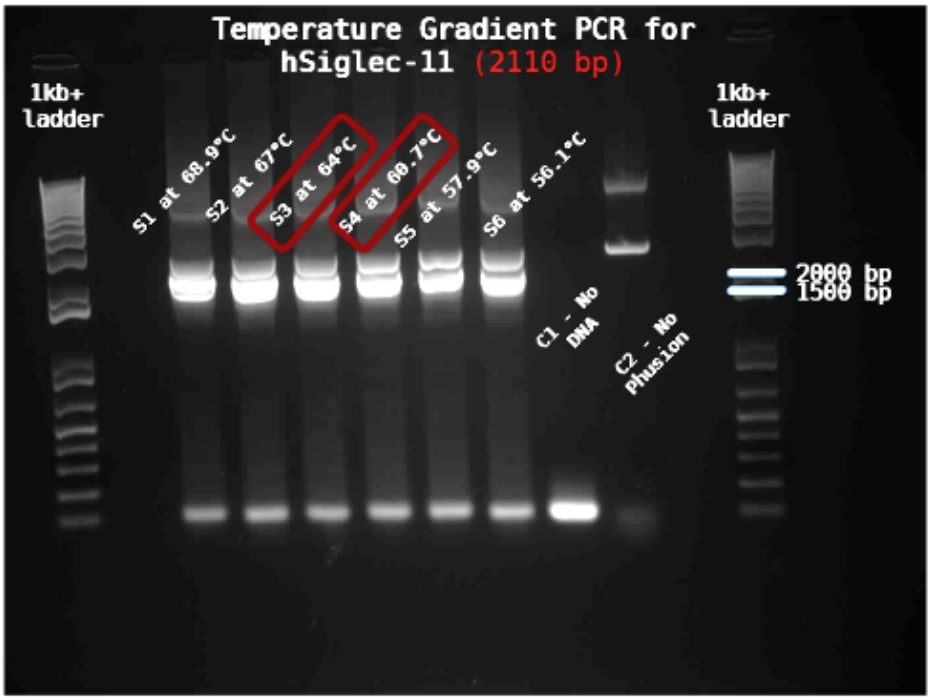


Figure 10-14. Verification of optimal annealing temperature for hSiglec11 PCR amplification. hSiglec11 was amplified at different annealing temperatures and ran on 1% agarose gel. The gel image shows the 1kb+ DNA molecular weight ladder with the 1.5kb and 2kb bands indicated; samples S1 to S6 indicate the amplifications of the hSiglec11 gene fragment; C1 and C2 are controls in the absence of either DNA or Phusion polymerase respectively.

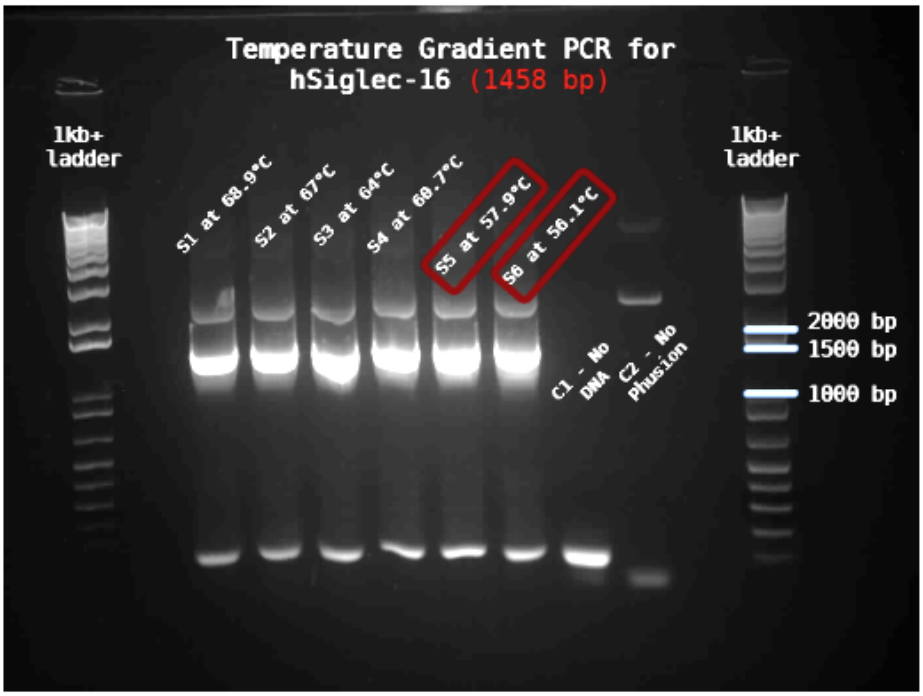


Figure 10-15. Verification of optimal annealing temperature for hSiglec16 PCR amplification. hSiglec16 was amplified at different annealing temperatures and ran on 1% agarose gel; the gel image shows the 1kb+ DNA molecular weight ladder with the 1kb, 1.5kb and 2kb bands indicated; samples S1 to S6 indicate the PCR amplifications of the hSiglec16 gene; C1 and C2 are controls in the absence of either DNA or Phusion polymerase respectively.

Figure 10-16 shows the verification of the optimal amplification of hSiglec11 and hSiglec16 fragments when visualized by agarose gel electrophoresis. The expected bands sizes of ~2.1kb and ~1.4kb were observed hSiglec11 and hSiglec16 respectively. Controls identical to the previous reactions (C1 and C2) were included. Small fade bands appear in both controls (C1 and C2) for hSiglec11 and hSiglec16. This might suggest the occurrence of primer dimerization within controls for the PCR amplification of the gene fragments of hSiglec11 and hSiglec16.

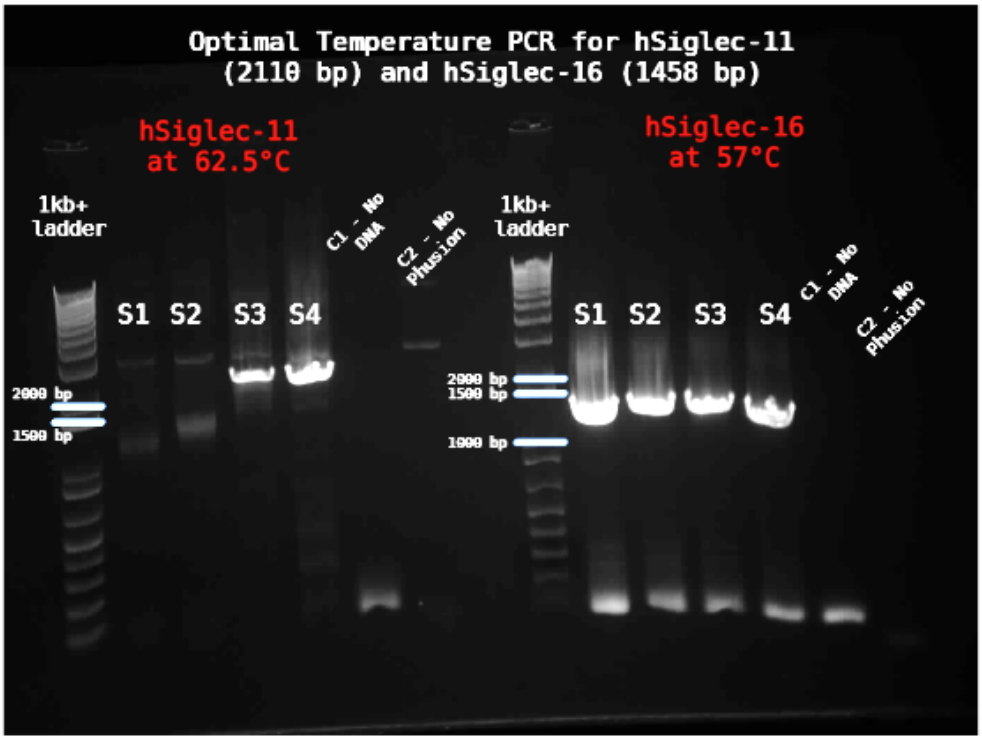


Figure 10-16. Analysis and confirmation of hSiglec11 and hSiglec16 PCR products. hSiglec11 and hSiglec16 fragments were amplified based on their optimal annealing temperature (marked in red); the 1kb+ ladder is indicated in the image with the 1bp, 1.5bp and 2bp band sizes; samples S1-S4 are the amplified fragments of hSiglec11/hSiglec16; C1 and C2 are the controls in the absence of either template DNA or Phusion polymerase.

The PCR products amplified as described above, were purified, and quantified with nanodrop. Final concentrations of the PCR products are shown in the Table 10-6 below. The DNA concentration for the pDONR221 was 240 ng/µl, for hSiglec11 was 270 ng/µl and for hSiglec16 was 690 ng/µl.

Table 10-6. DNA concentration of the purified PCR products and the isolated donor vector. Absorbance ratio at 260nm and 280nm ~1.8 verify the purity of the nucleic acids.

Sample ID Nucleic Acid	Concentration	Unit	$A_{260-280}$
pDONR221	240	ng/µl	1.80
hSiglec11	270	ng/µl	1.81
hSiglec16	690	ng/µl	1.70

10.11 Direct sequencing of the entry clone and the expression clones failed to verify the presence of GOIs

The gateway cloning is a time saving, highly efficient procedure that does not require the verification of the insert from both reactions BP and LR. However, a sequencing PCR was performed several times to verify the exchange/insert of the target gene fragments on the constructed entry clones from BP reaction and expression clones from LR reaction. No sequence reads were produced from the sequencing reaction.

10.12 Transfection of HEK293-TLR2 with the pEGFP-C2 was successful in almost 50% of the cells

The constructed pDEST YFP:hSiglec11 and pDEST YFP:hSiglec16 were transfected into HEK293-TLR2 cells in order to express these target proteins in their surface. To determine the efficiency of the transfection process, the pEGFP-C2 plasmid was used. This plasmid constitutively expresses the green fluorescent protein upon successful transfection into mammalian cells.

Figure 10-17 shows a captured field of the healthy transfected cells where no filter was applied while 10-18 shows a field image under the GFP filter verifying the efficiency of the transfection. Approximately 50% of the cells seem to contain the pEFGP-C2 plasmid.

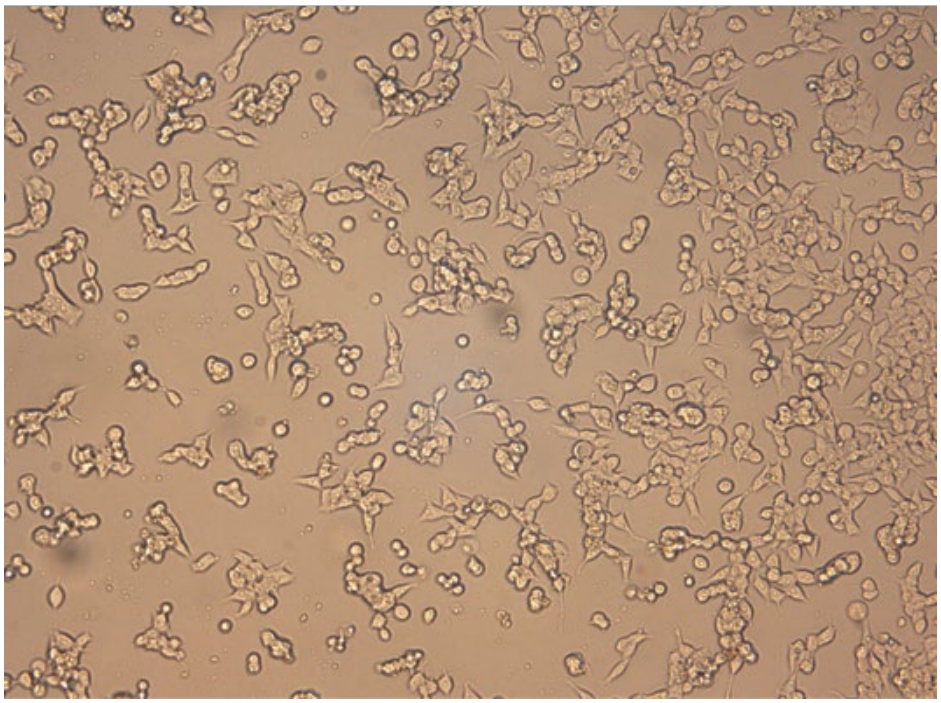


Figure 10-17. HEK293 cells transfected with the pEFGP-C2 plasmid. The image covers a field of the healthy transfected HEK293-TLR2 cells without the application of the GFP filter; the objective of the image is 10x and the concentration of the pEGFP-C2 was 5µg/µl.

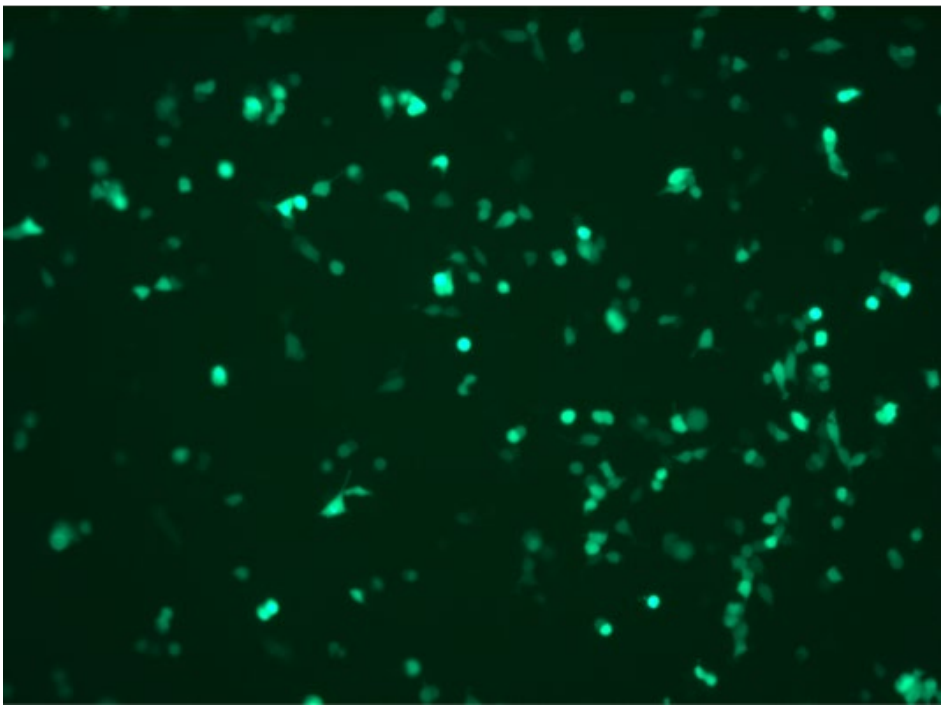


Figure 10-18. HEK293 cells transfected with the pEFGP-C2 plasmid. The image covers a field of the healthy transformed cells under the application of the GFP filter (green color); the green color indicates that around 50% of the cells have been transformed with the EFGP-C2 plasmid; objective: 10x; plasmid concentration: 5µg/µl.

10.13 Unusual high percentage of adhered *S. aureus* were observed in HEK293-TLR2 however, no bacteria were recovered from internalization and survival studies

To determine the biological implications of the interaction between *S. aureus* SdrD and the recombinant proteins hSiglec11 and hSiglec16, infection studies were performed. Non-transfected and transfected HEK293-TLR2 cells were infected with either NCTC8325-4 (Wild-Type) *S. aureus* strain or NCTC8325Δ*sdrD* (Knock-Out) *S. aureus* strain.

Figure 10-19 is a graphical presentation of the calculated data from the adhesion assay (Appendix 1). It shows the unusual high percentage of adhered *S. aureus* strains to the HEK293-TLR2 cells.

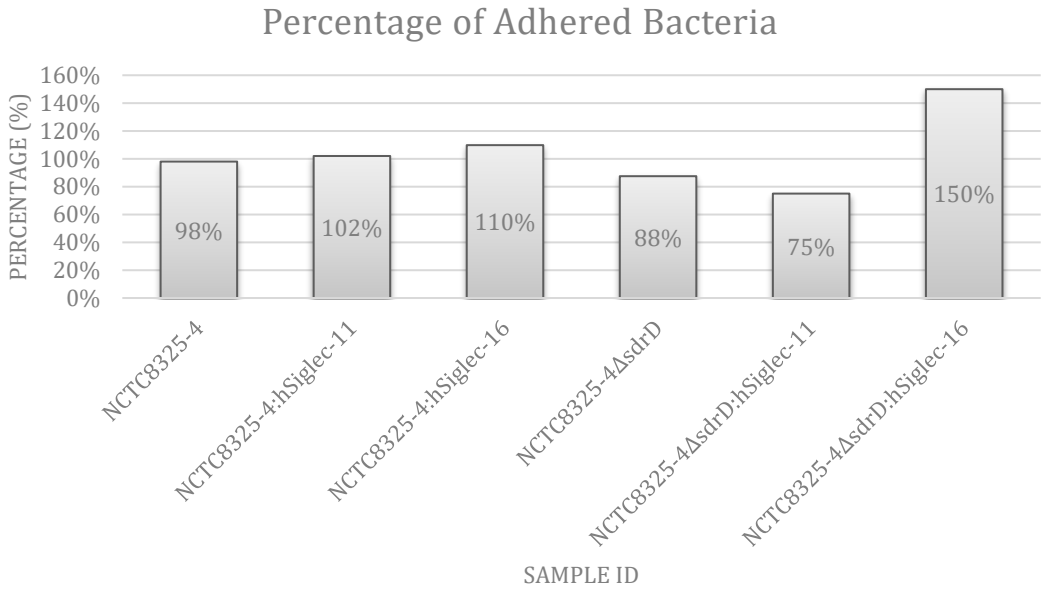


Figure 10-19. Percentage of adhered *S. aureus* in HEK293-TLR2. NCTC8325-4 and NCTC8325-4Δ*sdrD* are the non-transfected cells infected with the WT and KO strains respectively; NCTC8325-4:Siglec11(16) are the transfected cells with either Siglec11 or Siglec16 constructed plasmids infected with WT strain; NCTC8325-4 Δ*sdrD*:Siglec11(16) are the transfected cells with either Siglec11 or Siglec16 constructed plasmids infected with the KO strain.

10.14 LDH release measured during cytotoxicity assay in HEK293-TLR2 studies were mostly low indicating that the possible expression of Siglec11 and 16 proteins is not toxic

The raw data and the calculations to determine the LDH release during HEK293-TLR2 cells studies are present in Appendix 3 in details. The release of LDH was unusually higher during adhesion assay and negative values were calculated in internalization and survival assays.

10.15 Concentration of the IL-6 production measured during the ELISA assay resulted out of range for most samples

The concentration of IL-6 production for most samples was out of range. The raw data and calculations are in Appendix 5. Out of range values were mostly in HEK293-TLR2 cell studies. In THP1 cell studies, the concentration of IL-6 is higher after 5 hours and ON incubation in the presence of recombinant Siglec11 protein.

10.16 Studies on THP1 cells show no significant values in the infected cells with *S. aureus* in the absence/presence of recombinant Siglec11

The raw data of the infection studies performed on THP1 cells and the calculations are presented in Appendix 2. No data were recovered from the 2 hours and ON incubation assays. Table 10-7 indicated the calculated percentage of recovered bacteria after 5 hours incubation time point.

Table 10-7. Percentage of the recovered bacteria after 5 hours incubation in the absence/presence of Siglec11 in THP1 cells.

Infection Studies		5 Hours
Absence of recombinant Siglec11 in THP1	*NCTC8325-4*	*1%*
	NCTC8325-4ΔsdrD	*7%*
Presence of recombinant Siglec11 in THP1	*NCTC8325-4*	*1%*
	NCTC8325-4ΔsdrD	*10%*

LDH release during cytotoxicity assay in THP1 cells (raw data and calculations in Appendix 4 are presented as percentage values in Table 10-8. The release appears higher in the ON assay. The highest release was 27% in THP1 cells infected with isogenic mutant of *S. aureus*, in absence of recombinant Siglec11, in the ON assay.

Table 10-8. Percentage values of LDH release in THP1 cells.

	2 Hours	***5 Hours***	***Overnight***
NCTC8325-4	2%	3%	17%
NCTC8325-4:hSiglec11	3%	16%	17%
NCTC8325-4ΔsdrD	-3%	0%	27%
NCTC8325-4ΔsdrD:hSiglec11	-7%	-7%	9%
Negative Control	1%	-11%	4%
Positive Control	91%	95%	97%

11 Discussion

11.1 Brief introduction of the reported research

S. aureus infections are widely spread globally and the fact that its resistance towards antibiotic treatments is rapidly increasing supports the urgent need of an efficacious vaccine (79). The vaccine can be beneficial in decreasing its progression to severe diseases such as pneumonia or osteomyelitis, However, no licensed vaccine has yet been produced. Several vaccines which attack a single antigen have been unsuccessful as this sophisticated pathogen has found alternative ways to resist the host's immune responses thus, it is thought that an approach to develop a vaccine against multiple antigens could be more effective against S. aureus (80).

The Host-Microbe Interaction (HMI) research group of The Arctic University of Norway (UiT) is conducting research on *S. aureus* as a pathogen and one of its virulence factors termed SdrD present on its surface. SdrD has shown to increasingly affect *S. aureus* self-protective ways of survival against the host's immune system. The current study is a continuation of on-going unpublished research which identified several surface proteins present in plasma that seemed to have an interaction with the virulent factor SdrD which has already been shown to increase bacterial virulence and survival in blood (81). One of the proteins present in plasma that was identified to be a possible potential interaction partner with SdrD was Siglec16. However, more detailed research was needed to validate the possible potential interaction between them and its significance.

If there is an interaction between Siglec16 and the *S. aureus* SdrD, could this help the immune cells in identifying, fighting, and eradicating this pathogen? Or does *S. aureus* use the interaction in its favor to escape, survive and replicate inside the host's immune cells?

The biggest challenge during the research was the incomplete knowledge on the family of Siglecs. These proteins mostly co-function in pairs. The pairs have opposite inflammatory functions which commonly has resulted in antibody cross-reaction, making it hard to learn more about their structure, biological functions, and binding partners.

11.2 Methodological challenges

A series of methodological and technical challenges were encountered during the research thus, making it difficult to properly reach the aims of the study however, helpful observations were made that could optimize several processes for achieving conclusive and/or significant results in further research projects on *S. aureus* SdrD and Siglec proteins.

11.2.1 Expression of hSiglec16 full and hSiglec16 extra in Lemo21 (DE3) competent cells

One of the aims was the purification of the Human Siglec16 for use on biological and structural function analysis to elucidate and validate its interaction with *S. aureus* SdrD as the recombinant protein for Human Siglec16 was commercially unavailable for purchase in August 2020. Therefore, the Human Siglec16 Versaclone cDNA was purchased from RnD Systems to clone its ORF in an expression prokaryotic vector termed pT7-Flag-2. This is a ~4.8kb *E. coli* expression vector used for cloning and cytoplasmic expression of ORFs as a C-terminal Flag Fusion protein.

The Flag-fusion protein can be detected by the Monoclonal Anti-Flag® M2 and purified using the Anti-Flag M2 Affinity Gel. The vector requires the use of *E. coli* cells that contain a source of T7 polymerase. The T7 promoter region drives the transcription of the hSiglec16-Flag Fusion constructs. Therefore, the Lemo21 (DE3) competent cells engineered for an optimal expression of transmembrane proteins such as hSiglec16, were the chosen *E. coli* strain to carry out the expression studies.

Human Siglec16 is a transmembrane protein with a calculated size of ~54 kDa. The ORFs of the full-length hSiglec16 and its extracellular domain were inserted into the chosen prokaryotic expression vector during a traditional cloning procedure. The overall traditional cloning results verified the successful insert of the ORFs in the final construct pT7-Flag-2:hSiglec16 extra. The ORF insert for the full length hSiglec16 failed as the results obtained from the direct sequencing were inconclusive. Some technical difficulties were encountered during the procedure. Quantification of the plasmid DNAs isolated from the transformed DH5α cells, resulted in high concentrations, probably as they were extracted using the low-copy plasmid isolation protocol suggesting that pT7-Flag-2 and phSiglec16 are in fact high-copy plasmids. The absorbance ratio at 260nm and 280nm have values close to the 1.8 ration which is generally accepted as ‘pure’ for nucleic acids. Lower rations could indicate possible reagent contamination used in the isolation process.

Furthermore, the GOIs were amplified with a PCR reaction at an optimal annealing temperature for each of them. Visualization with agarose gel electrophoresis confirmed the success of the PCRs performed as the obtained bands were of expected sizes. However, several small and faded bands were observed in the control samples from some of the agarose gel images of hSiglec16 full. The presence of these bands can be an indication of contaminated samples. Contamination can happen during the preparation of the reaction mixes, during pipetting of the reagents into the PCR tubes or it can be in reagents used in PCR. The latter hypothesis can only be valid only for the contamination of the Fwd and Rwd primers of the hSiglec16 full primers, as the other components were used in the amplification of hSiglec16 extra where the control samples appear clear of unexpected bands. Another suggestion based on the small band sizes, is the dimerization of primer molecules which if present in high concentrations can link to other primer molecules and appear as dull, small size bands.

In the ligation process, the products in the agarose gel image appear larger than the expected size however, considering that the product lanes contain the whole digested amount of the purified PCR products in high concentrations, running the gel for a longer period of time could have provided a better visualization of the bands in their expected sizes. In addition, the 1kb+ weight molecular ladder is not completely stretched out and clearly visible, which can justify the larger appearance of the target bands and suggests a problem might be the insufficient current during electrophoresis. This result was accepted as successful based on the above hypothesis and on the fact that each product lane contains only one band. This strongly supports the identity and purity of the samples ran on 1% agarose gel.

The biggest challenge in the protein expression studies was verifying and optimizing the expression of target proteins. Sonication of the samples was an update made due to in solubilization of the samples making it impossible to separate the soluble and insoluble fractions. Coomassie staining of the SDS-PAGE gels was performed several times in order to detect the target proteins however, it always failed. The lysate samples ran on gel were too concentrated to detect specific protein bands. If the affinity gel for the Flag-fusion protein was used to purify the lysate samples before running them in the gel, then the results could have been more specific towards the detection of the targets.

The next approach in target identification was blotting the lysate samples using the Monoclonal Anti-Flag M2 antibody produced in mouse that can detect the Flag-tag present in the C-terminal of the constructed expression vectors. The specific monoclonal antibody detected similar flag-

tag sequences, as several bands appeared in the scanned membrane making it even harder and confusing to conclude the success or failure of the expression of the GOIs. However, a study on gene tagging strategies suggests that a tag should be incorporated after a START codon or before a STOP codon (82). Realization of the missing STOP codon after the Flag-tag suggested that the protein bands can appear larger than expected. This redirected the research towards more specific protein identification approaches such as the use of the hSiglec16 antibody.

The commercial antibody for the Human Siglec16 became available for purchase during the research. Western Blot using this specific antibody was performed on newly prepared bacterial lysates in alternative growing conditions for the bacterial expression cultures considering the previous experienced obstacles and that the target proteins might be toxic to the Lemo21 cells for period of times 5-hours and overnight. The bacterial cultures were induced for 3-hours and 5-hours, extracted and blotted using the specific antibody of hSiglec16. In the 3h time-point bands in the expected size of ~44kDa for the hSiglec16 extra were noticed in the uninduced-unregulated samples and in the induced samples with IPTG in absence of rhamnose. The band is more integrated in the soluble fraction however, traces of the protein were seen in the expected size, induced with IPTG in the insoluble fraction supporting the reliability of these observations.

Neither of the optimalizations of growth conditions was positive in increasing expression of GOIs in the *E. coli* cells. The limited research time for completing the aims of the study based on several delays due to the global pandemic of 2020/2021, this objective was not completed.

11.2.2 Construction of mammalian expression vectors of hSiglec11 and hSilgec16 using pDest YFP gateway cloning

Gateway cloning is a time-saver, highly efficient cloning procedure. Its reactions incorporate the GOIs though region exchange using specific attB primers for the insert of the DNA of interest. The validation of the isolated constructs pDEST YFP:hSiglec11 and pDEST YFP:hSiglec16, by direct sequencing was unsuccessful. The sequencing reactions were performed more than 5x, in entry and expression clones from BP and LR reactions. However, the reads remained unidentifiable, short, or empty of any sequence. Based on all sequencing PCR made in the study, the suggested issue was the unreadable plasmid sequence during the procedure.

11.2.3 *S. aureus* infection studies on HEK293-TLR2 cells transfected with the recombinant hSiglec11/hSiglec16

The successful transfection of the mammalian HEK293-TLR2 cells with the pEGFP-C2 indicated an efficiency of ~50%. Thus, considering the inconclusive gateway cloning results positive, the transfection of the cells with the LR expression clones for hSiglec11 and hSiglec16 was ~50% efficient.

No bacteria were recovered in internalization and survival studies of HEK293-TLR2 cells. If the original undiluted collected supernatants were plated out from these assays, the results would have been slightly different. The adhesion assay resulted in unusual high percentage of adhered *S. aureus* strains in all HEK293-TLR2 cells, transfected or non-transfected. Therefore, an interaction between SdrD and hSiglec11 or SdrD and hSiglec16 was not observed. Another hypothesis based on the similar percentages can indicate the absence of these target proteins in cells. The LDH release of the supernatants collected from the adhesion assay had unreliable values as the negative controls seem to have ~100% release of LDH while positive controls ~200%. The LDH release in internalization and survival assay resulted in negative values strongly supporting the unreliability of the assay.

11.2.4 *S. aureus* infection in THP1 cells in the presence of recombinant hSiglec11

The infection studies on THP1 cells present low percentage of survived bacteria after 5 hours incubation in the presence or absence of recombinant hSiglec11 protein. The 2 hours and ON assays had no survived bacteria. Less than ~30% LDH release was noticed in the ON supernatants showing that most of the cells were healthy. However, the values are not significantly comparable to conclude in the effect of the presence or absence of the hSiglec11 during infection with *S. aureus* strains.

11.2.5 IL-6 concentration in infection studies of HEK293-TLR2 and THP1 cells

Interleukin-6 is an early inflammatory marker of complicated *S. aureus* bloodstream infections (83). *S. aureus* can regulate the secretion of IL-6 by activating nuclear factor kappaB in human osteoblasts (84). The IL-6 concentration in HEK293-TLR2 cells were out of range in internalization and survival assays while in THP1 cells the concentrations are higher in the ON assay. The highest concentration is in the cells infected with WT strain of *S. aureus* in absence (87.2 pg/ml) and presence (88 pg/ml) of Siglec11 protein. As these values are highly similar, it indicates that the presence of Siglec11 has no significant role in relation to *S. aureus* infection.

12 Conclusion

The infection studies which support the main research goal of this project was conducted once due to the limitation of time thus, making the overall observations unreliable to give a clear conclusion on the interaction between SdrD of *S. aureus* and the Siglec proteins present in cell surfaces. Future perspectives in achieving a better insight on their interaction and where does that lead to, is successfully achieving all the crucial stages of this study. Repetition and alternation of the procedures is important in obtaining reliable outcomes. One of them is verifying and optimizing the expression of the Siglec proteins in cells by Western Blot using their specific antibodies.

13 Appendix

13.1 Appendix 1

The following tables show the raw data and calculations made based on obtained results from the assays performed on HEK293-TLR2.

CFU/ml was calculated according to formula:

CFU/ml = (nr. of colonies x Dilution Factor) / Plated Volume

Table 13.1-6. Raw data obtained from the plating of the inoculum. The dilution factor was 1000. There were 6 parallels plated for each strain in a volume of 25µl. The expected MOI was 1.

INOCULUM	Wild-Type		Knock-Out
	NCTC8325-4		NCTC8325-4ΔSdrD
Dilution Factor	1000		1000
CFU count	5		8
CFU count	8		7
CFU count	6		6
CFU count	6		9
CFU count	13		10
CFU count	13		8
Vol. Plated (ml)		0.025	
Average	8.5		8
CFU/ml	340000		320000
Seeded Cells	150000		150000
Counted Cells	180000		180000
Expected MOI	1		1
Actual MOI	1.9		1.8

Table 13.1-7. Raw data collected from the adhesion assay performed on HEK293-TLR2 cells. WT represents the Wild-Type strain of S. aureus while KO the Knock-out strain for sdrD. Numbers 11 and 16 represent the cells transfected with the corresponding Siglec proteins 11 or 16.

ADHESION						
	WT	WT + 11	WT + 16	KO	KO + 11	KO + 16
Dilution Factor	1000	1000	1000	1000	1000	1000
CFU count	9	11	9	7	6	22
CFU count	8	6	11	7	8	8
CFU count	11	5	9	7	6	7
CFU count	6	12	9	3	4	13
CFU count	10	8	8	9	7	12
CFU count	6	10	10	9	5	10
Vol. Plated (ml)	0.025					
Average	8.3	8.7	9.3	7	6	12
CFU/ml	333333	346667	373333.3	280000	240000	480000

The percentage of the adhered bacteria from the adhesion assay was calculated as follows:

Percentage of Adhered Bacteria = (CFU/ml of Adhered / CFU/ml of Inoculum) x 100

Table 13.1-8. Percentage of the adhered bacteria in HEK293-TLR2 cells.

Sample ID	Percentage of Adhered Bacteria
NCTC8325-4	*98%*
NCTC8325-4:hSiglec-11	*102%*
NCTC8325-4:hSiglec-16	*110%*
NCTC8325-4ΔsdrD	*88%*
NCTC8325-4ΔsdrD:hSiglec-11	*75%*
NCTC8325-4ΔsdrD:hSiglec-16	*150%*

Table 13.1-9. Raw data collected from the internalization assay on HEK293-TLR2 cells. WT represents the Wild-Type strain of S. aureus while KO the Knock-out strain for sdrD. Numbers 11 and 16 represent the cells transfected with the corresponding Siglec proteins 11 or 16.

INTERNALIZATION						
	WT	**WT + 11**	**WT + 16**	**KO**	**KO + 11**	**KO + 16**
Dilution Factor	1000	1000	1000	1000	1000	1000
CFU count	0	0	0	0	0	0
Average	0	0	0	0	0	0
CFU/ml	0	0	0	0	0	0

Table 13.1-10. Raw data collected from the survival assay of HEK293-TLR2 cells. WT represents the Wild-Type strain of S. aureus while KO the Knock-out strain for sdrD. Numbers 11 and 16 represent the cells transfected with the corresponding Siglec proteins 11 or 16.

SURVIVAL						
	WT	**WT + 11**	**WT + 16**	**KO**	**KO + 11**	**KO + 16**
Dilution Factor			100			
CFU count			8			
CFU count			6			
CFU count			5			
CFU count			6			
CFU count			9			
CFU count			8			
Vol. Plated (ml)	0.025					
Average			7			
CFU/ml			28000			

13.2 Appendix 2

The following tables show the raw data and calculations made based on obtained results from the assays performed on THP1 cells.

Table 13.2-11. Raw data obtained from the plating of the inoculum. The dilution factor was 10000. The expected MOI was 5 however the actual one appeared lower. WT represents the Wild-Type strain of S. aureus while KO the Knock-out strain for sdrD. The number 11 represents the cells where the recombinant Siglec11 protein was added during the infection with S. aureus strains.

INOCULUM					
		WT	WT + 11	KO	KO + 11
Dilution Factor		10000	10000	10000	10000
CFU count		8	9	8	9
CFU count		6	11	11	9
CFU count		8	10	13	10
CFU count		9	12	6	6
CFU count		7	13	14	15
CFU count		8	12	12	9
Vol. Plated (ml)	0.025				
Average		7.7	11.2	10.7	9.7
CFU/ml		3066667	4466667	4266667	3866667
Seeded Cells		500000	500000	500000	500000
Counted Cells		1250000	1250000	1250000	1250000
Expected MOI		5	5	5	5
Actual MOI		2.5	3.6	3.4	3.1

Percentage of the survived bacteria after specific time points was calculated as shown below:

Percentage of Survived Bacteria = (CFU/ml of Survived / CFU/ml of Inoculum) x 100

Table 13.2-12. Raw data collected after the 5 hours assay performed on THP1 cells. ***WT represents the Wild-Type strain of*** **S. aureus** ***while KO the Knock-out strain for*** **sdrD.** ***The number 11 represents the cells where the recombinant Siglec11 protein was added during the infection with*** **S. aureus** ***strains. The percentage of survived bacteria was calculated according to the inoculum in table 13.2-1.***

5 HOURS TIME-POINT					
		WT	**WT + 11**	**KO**	**KO + 11**
Dilution Factor		100	100	1000	1000
CFU count		5	15	9	13
CFU count		7	9	8	8
CFU count		3	14	6	10
CFU count		3	15		7
CFU count		4	17		9
CFU count		4	16		9
Vol. Plated (ml)	0.025				
Average		4.3	14.3	7.7	9.3
CFU/ml		17333	57333	306667	373333
Percentage		1%	1%	7%	10%

13.3 Appendix 3

Data from the cytotoxicity assay using the samples collected from the assays performed on HEK293-TLR2 cells.

Table 13.3-13. The format of the plate where the cytotoxicity assay was performed. WT represents the Wild-Type strain of S. aureus while KO the Knock-out strain for sdrD. Numbers 11 and 16 represent the cells transfected with the corresponding Siglec proteins 11 or 16. HEK represents the non-transfected cells.

	ADHESION			INTERN			SURVIV		
	16	**11**	**HEK**	**16**	**11**	**HEK**	**16**	**11**	**HEK**
Blank	WT	WT	WT	WT	WT	WT	WT	WT	WT
Blank	WT	WT	WT	WT	WT	WT	WT	WT	WT
	KO	KO	KO	KO	KO	KO	KO	KO	KO
	KO	KO	KO	KO	KO	KO	KO	KO	KO
	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Neg	Neg	Neg	Poz	Neg	Neg	Neg	Neg	Neg
	Poz	Poz	Poz	Poz	Poz	Poz	Poz	Poz	Poz
	Poz	Poz	Poz	Neg	Poz	Poz	Poz	Poz	Poz

Table 13.3-14. The observed raw data of the assay. Numbers 11 and 16 represent the cells transfected with the corresponding Siglec proteins 11 or 16. HEK represents the non-transfected cells.

		ADHESION			INTERN			SURVIV		
Temp		**16**	**11**	**HEK**	**16**	**11**	**HEK**	**16**	**11**	**HEK**
23.2	0.65	0.97	1.32	2.07	0.60	0.49	0.47	1.50	1.58	0.59
	0.63	1.02	1.15	2.41	0.46	0.36	0.34	0.80	0.64	0.52
		1.62	1.29	0.92	0.31	0.53	0.41	0.50	2.50	1.22
		1.16	1.28	2.05	0.35	0.58	0.38	0.45	1.23	0.62
		2.08	1.46	1.58	0.48	0.42	0.37	0.57	0.50	0.59
		1.37	2.73	1.69	2.06	0.35	0.36	0.48	0.59	0.53
		3.13	2.47	3.44	1.76	1.93	2.19	2.16	1.49	2.09
		2.49	2.57	3.25	0.55	1.25	1.46	2.20	1.27	2.12

Table 13.3-15. The calculated average data minus the background (blank) of the samples. Numbers 11 and 16 represent the cells transfected with the corresponding Siglec proteins 11 or 16. HEK represents the non-transfected cells.

		ADHESION			INTERN			SURVIV		
		16	**11**	**HEK**	**16**	**11**	**HEK**	**16**	**11**	**HEK**
Temp	0	0.35	0.59	1.60	-0.12	-0.22	-0.24	0.51	0.47	-0.08
23.2		0.74	0.64	0.84	-0.31	-0.09	-0.24	-0.17	1.22	0.28
		1.08	1.45	0.99	0.57	-0.26	-0.28	-0.12	-0.10	-0.08
		2.17	1.88	2.70	1.27	0.95	1.18	1.53	0.74	1.46

Table 13.3-16. Data from the cytotoxicity assay performed on HEK293-TLR2.

	Adhesion	**Internalization**	**Survival**
NCTC8325-4	1.6	-0.2	-0.1
NCTC8325-4ΔsdrD	0.8	-0.2	0.3
Negative Control	1.0	-0.3	-0.1
Positive Control	2.7	1.2	1.5
NCTC8325-4:hSiglec11	0.6	-0.2	0.5
NCTC8325-4ΔsdrD:hSiglec11	0.6	-0.1	1.2
Negative Control	1.5	-0.3	-0.1
Positive Control	1.9	0.9	0.7
NCTC8325-4:hSiglec16	0.4	-0.1	0.5
NCTC8325-4ΔsdrD:hSiglec16	0.7	-0.3	-0.2
Negative Control	1.1	0.6	-0.1
Positive Control	2.2	0.5	1.5

13.4 Appendix 4

Data from the cytotoxicity assay using the samples collected from the assays performed on THP1 cells.

Table 13.4-17. The format of the plate where the cytotoxicity assay was performed. WT represents the Wild-Type strain of S. aureus while KO the Knock-out strain for sdrD. The number 11 represents the cells where the recombinant Siglec11 protein was added during the infection with S. aureus strains.

	2 Hours			5 Hours			Overnight		
Blank	Neg	WT	KO	Neg	WT	KO	Neg	WT	KO
Blank	Neg	WT	KO	Neg	WT	KO	Neg	WT	KO
	Poz	WT + 11	KO + 11	Poz	WT + 11	KO + 11	Poz	WT + 11	KO + 11
	Poz	WT + 11	KO + 11	Poz	WT + 11	KO + 11	Poz	WT + 11	KO + 11

Table 13.4-18. The observed raw data from the assay.

Temp	2 Hours				5 Hours			Overnight		
22.7	0.44	0.45	0.52	0.46	0.34	0.49	0.50	0.55	0.61	0.84
	0.44	0.46	0.39	0.36	0.32	0.44	0.39	0.41	0.61	0.59
		1.56	0.54	0.38	1.56	0.68	0.38	1.64	0.60	0.52
		1.14	0.40	0.36	1.21	0.51	0.37	1.17	0.62	0.53

Table 13.4-19. The calculated average data minus the background (blank).

Temp	2 Hours				5 Hours			Overnight		
22.7	0.00	0.01	0.02	-0.03	-0.11	0.03	0.00	0.04	0.17	0.27
		0.91	0.03	-0.07	0.95	0.16	-0.07	0.97	0.17	0.09

Table 13.4-20. Cytotoxicity percentage based on the values from table 13.4-3.

	2 Hours	*5 Hours*	*Overnight*
NCTC8325-4	2%	3%	17%
NCTC8325-4:hSiglec11	3%	16%	17%
NCTC8325-4ΔsdrD	-3%	0%	27%
NCTC8325-4ΔsdrD:hSiglec11	-7%	-7%	9%
Negative Control	1%	-11%	4%
Positive Control	91%	95%	97%

13.5 Appendix 5

Data from ELISA assay performed on HEK293-TLR2 internalization and survival assays and on THP1 cells.

Table 13.5-21. The format of the plate where ELISA assay was performed. On HEK293-TLR2 cells the samples collected from internalization and survival assays were used. WT represents the Wild-Type of S. aureus strain while the KO is the Knock-Out strain for sdrD. Numbers 11 and 16 represent the HEK cells transfected with one of the Siglec proteins 11 or 16 while HEK represent the non-transfected cells. On THP1 cells, samples collected from all three assays were used. Number 11 represents the cells where the recombinant Siglec11 protein was added during the infection with one of S. aureus strains.

	HEK293			HEK293			THP1			
	INT.			SURV.						
Standard	16	11	HEK	16	11	HEK	2 Hours	5 Hours	ON	Negative
	WT	WT	WT	WT	WT	WT	WT	WT	WT	at 2h
	WT	WT	WT	WT	WT	WT	WT	WT	WT	
	KO	KO	KO	KO	KO	KO	WT + 11	WT + 11	WT + 11	at 5h
	KO	KO	KO	KO	KO	KO	WT + 11	WT + 11	WT + 11	
	Neg	Neg	Neg	Neg	Neg	Neg	KO	KO	KO	ON
	Neg	Neg	Neg	Neg	Neg	Neg	KO	KO	KO	
							KO + 11	KO + 11	KO + 11	
							KO + 11	KO + 11	KO + 11	

Table 13.5-22. Raw data of ELISA assay after wavelength correction.

		HEK293			HEK293			THP1			
		INT.			SURV.						
Stnd.	Stnd.	16	11	HEK	16	11	HEK	2 Hours	5 Hours	ON	Negative
0.0	0.0	0.04	0.04	0.09	0.19	0.07	0.11	0.03	1.89	3.76	0.04
0.2	0.2	0.03	0.02	0.04	0.06	0.09	0.09	0.03	1.96	3.82	
0.3	0.3	0.04	0.05	0.05	0.08	0.08	0.09	0.03	2.33	3.84	0.06
0.6	0.6	0.04	0.05	0.04	0.07	0.08	0.08	0.03	2.38	3.79	
1.1	1.1	0.04	0.04	0.04	0.06	0.07	0.07	0.02	0.74	3.85	0.06
1.9	2.0	0.05	0.05	0.05	0.07	0.08	0.08	0.02	0.07	1.54	
3.2	3.2							0.01	0.10	2.57	
3.6	3.7							0.02	0.10	2.70	

Table 13.5-23. The average data based on the wavelength correction.

	HEK293			HEK293			THP1		
		INT.			SURV.				
Stnd.	**16**	**11**	**HEK**	**16**	**11**	**HEK**	**2 Hours**	**5 Hours**	**ON**
0	0.04	0.03	0.06	0.13	0.08	0.10	0.03	1.92	3.79
0.2	0.04	0.05	0.04	0.07	0.08	0.08	0.03	2.36	3.82
0.3	0.04	0.05	0.05	0.06	0.07	0.08	0.02	0.40	2.70
0.6							0.01	0.10	2.63
1.1							0.04	0.06	0.06
2									
3.2									
3.7									

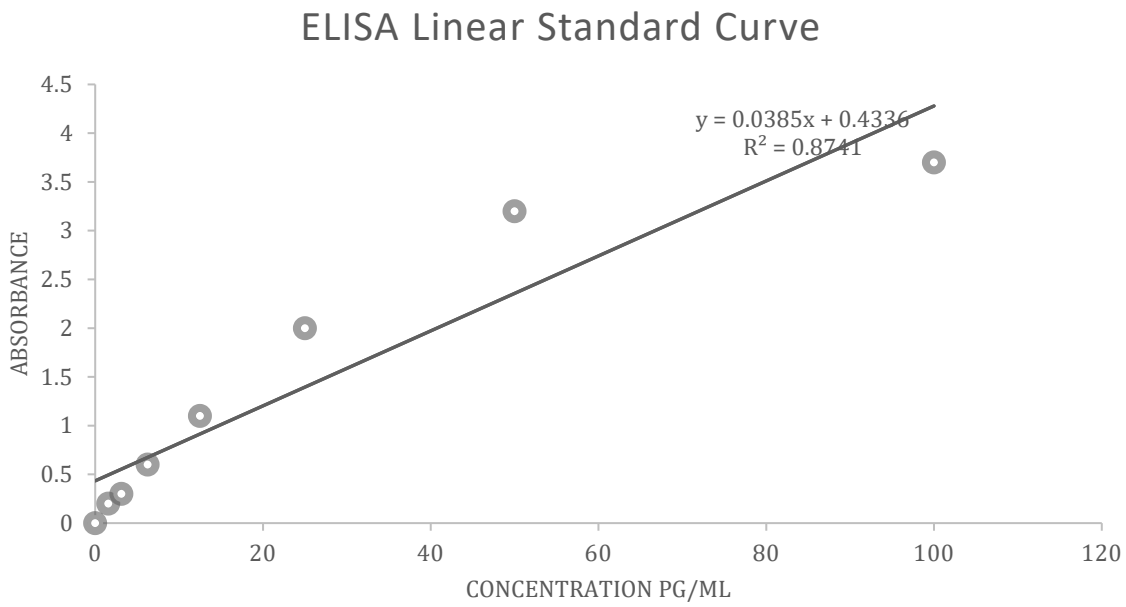


Figure 13-20. Linear Standard Curve for ELISA assay. The equation and R^2 value are shown in the graphic.

Table 13.5-24. Concentration of interleukin 6 in HEK293-TLR2 cells. The minus sign represents negative values

HEK293-TLR2	*Internalization*	*Survival*
NCTC8325-4	-	-
NCTC8325-4ΔsdrD	-	-
Negative Control	-	-
NCTC8325-4:hSiglec11	-	-
NCTC8325-4ΔsdrD:hSiglec11	-	-
Negative Control	-	-
NCTC8325-4:hSiglec16	-	-
NCTC8325-4ΔsdrD:hSiglec16	-	-
Negative Control	-	-

Table 13.5-25. Concentration of interleukin 6 in THP1 cells. The minus sign represents negative values.

THP1	*2 Hours*	*5 Hours*	*Overnight*
NCTC8325-4	-	38.6	87.2
NCTC8325-4:Siglec11	-	50.0	88.0
NCTC8325-4ΔsdrD	-	-	58.9
NCTC8325-4ΔsdrD:Siglec11	-	-	57.0
Negative Control	-	-	-

13.6 Appendix 6

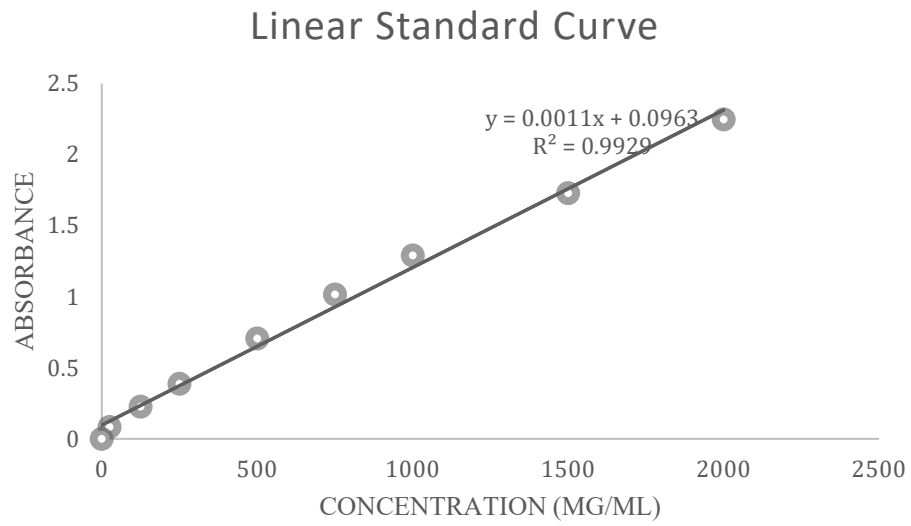


Figure 13-21. The standard linear curve from Bradford assay.

The equation from the standard curve was used to calculate the average concentration of the Siglec16 protein present in lysed bacteria cells during the small-scale expression studies.

The absorbance of the unknown sample was: 3.544 therefore the average concentration for the Siglec16 protein was calculated around 3134 µg/ml. This value was used to dilute the samples into a smaller concentration, in order to observe defined bands in the downstream analysis.

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